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A MANUAL

OF

CLINICAL CHEMISTRY, MICROSCOPY, AND BACTERIOLOGY

A MANUAL
OF
CLINICAL CHEMISTRY, MICROSCOPY, AND BACTERIOLOGY

BY

DR. M. KLOPSTOCK AND DR. A. KOWARSKY
OF BERLIN

In their "Institut für medizinische Diagnostik," in Berlin

ONLY AUTHORIZED TRANSLATION FROM THE LAST
GERMAN EDITION, THOROUGHLY REVISED
AND ENLARGED

*ILLUSTRATED WITH FORTY-THREE TEXTUAL FIGURES
AND SIXTEEN COLORED PLATES*

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PREFACE TO THE GERMAN EDITION

This book owes its existence to the desire of the authors to place a concise manual in the hands of those taking part in the course in Clinical Chemistry, Microscopy, and Bacteriology held in their "Institut für medizinische Diagnostic," in Berlin. It is in nowise intended to replace larger and more elaborate text-books, but aims to present in concise form the essential features of the subjects treated. As the book is intended especially for the practitioner, we have assumed that the reader has an elementary chemical and bacteriological education. For the same reason the needs of daily practice have been especially considered in the choice of the methods of examination. Wherever it has been possible the simplest and quickest methods have been chosen.

It would be a source of gratification to us if this book should find favor in wider medical circles.

The Authors.

248545

PUBLISHERS' ANNOUNCEMENT

Having found this book a valuable laboratory guide, it is a pleasure to comply with the request of the Authors to publish an English Translation of this New Edition. The Authors have retained all those matters which by experience have proved themselves to be of genuine, practical value to the student as well as to the medical man in General Practice. The pages relating to Typhoid Fever and to the Meningococci have been rewritten with great care; whilst those dealing with the Spirocheta pallida and with the *Wassermann* Reaction, especially, are entirely new.

The book has been brought up to date. The Index at the end of the volume is a new feature, and the references to the pages where they are quoted will prove of real value when consulting the colored plates.

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CONTENTS

CHAPTER I

BACTERIOLOGICAL EXAMINATION OF THE SECRETIONS AND DEPOSITS IN THE MOUTH AND PHARYNX

PAGE
Collection of Material to be Examined 1
Morphological and Staining Characteristics of Diphtheria Bacilli 2
Cultural Characteristics.............................. 4
Animal Inoculation.................................. 5
Differential Diagnosis.............................. 5
Order of Examination................................ 8
Oidium albicans —"Soor fungus"........................ 10
Angina Vincenti (s. Plautii).......................... 11

CHAPTER II

BACTERIOLOGICAL EXAMINATION OF NASAL SECRETIONS

Diphtheria Bacilli.................................. 17
Tubercle Bacilli.................................... 17
Lepra Bacilli....................................... 18
Diplobacillus of *Friedlaender*.......................... 18

CHAPTER III

BACTERIOLOGICAL EXAMINATION OF CONJUNCTIVAL SECRETION

Diphtheria Bacilli.................................. 19
Tubercle Bacilli.................................... 19
Gonococci 20
Bacilli of *Koch* and *Weeks*.......................... 20
Diplobacillus of *Morax* and *Axenfeld*..................... 21
Other Exciters of Conjunctivitis........................ 21

CHAPTER IV

EXAMINATION OF THE SPUTUM

PAGE
Method of Obtaining Material for Examination 22
General Characteristics.................................. 23
Especially Prominent Constituents of the Sputum......... 23
Composition of Sputum.................................. 24
Especially Prominent Ingredients of the Sputum.......... 26
Microscopical Examination.............................. 28
    Curschmann's Spirals............................. 29
    Fibrin Coagula..................................... 30
    Dittrich's Plugs................................. 30
    Portions of the Echinococcus....................... 31
    Actinomyces Granules............................... 32
Cellular Elements of the Sputum........................ 33
Elastic Fibres... 35
Crystalline Bodies 36
Bacteriological Examination of the Sputum.............. 37
    Examination of Stained Smears...................... 38
    Cultural Examination............................... 40
    Animal Inoculation 40
    Detection of Tubercle Bacilli...................... 40
Pneumococci.. 48
Streptococci 49
Staphylococci 50
Micrococcus tetragenus............................... 50
Micrococcus catarrhalis.............................. 51
Influenza Bacillus..................................... 51
Diplobacillus of *Friedlaender* 52
Bacillus pyocyaneus.................................. 53
Bacillus of Bubonic Plague............................. 53

CHAPTER V

EXAMINATION OF THE GASTRIC CONTENTS

General Characteristics................................ 56
Qualitative Chemical Examination....................... 58
    Reaction... 58

PAGE
Qualitative Chemical Examination:
Free Acids.. 58
Free Hydrochloric Acid.......................... 58
Lactic Acid....................................... 61
Volatile Fatty Acids.............................. 61
Pepsin and Pepsinogen............................ 62
Renin and Reninogen.............................. 65
Bile Pigment...................................... 66
Blood... 66
Hydrogen Sulphide................................ 68
Quantitative Chemical Examination of the Gastric Contents... 68
Estimation of Total Acidity....................... 68
" of Free Hydrochloric Acid.................... 69
" of Total Hydrochloric Acid................... 70
" of Lactic Acid............................... 71
Microscopical Examination of the Gastric Contents....... 72

CHAPTER VI

EXAMINATION OF THE FÆCES

General Characteristics.......................... 74
Qualitative Chemical Examination of the Fæces........... 80
Reaction.. 80
Mucin... 80
Fat... 81
Blood... 81
Biliary Constituents.............................. 82
Quantitative Chemical Examination of the Fæces......... 84
Estimation of Dry Matter.......................... 84
" of Total Nitrogen............................ 85
" of Fat....................................... 85
" of Carbohydrates............................. 86
Direct Estimation of Starch According to *Liebermann* and *Allihn*.. 87
Fermentation Test According to *Schmidt*.................. 88
Examination of Gall-Stones and Biliary Concretions....... 90
Fecal Concretions, Enteroliths, and Pancreatic Stones.... 92

PAGE
Microscopical Examination of the Fæces 94
    Food Particles 94
Pathological Products of the Intestinal Wall 96
Intestinal Parasites and their Eggs 97
Bacteriological Examination of the Fæces 104
Typhoid Bacilli 104
    Characteristics of Typhoid Bacilli 105
Biological Characteristics of Typhoid Bacilli 108
Order of Examination of the Fæces for Typhoid Bacilli 113
Planting of Cultures from the Fæces 113
Examination of the Plates 114
Bacilli in Meat-Poisoning 117
Dysentery Bacilli 117
Cholera Vibriones 120
    Pfeiffer's Test 122
    Detection of Cholera Vibriones in the Fæces 125
Tubercle Bacilli 126
Staphylococci and Streptococci 127
Anthrax Bacilli 128
Plague Bacilli 128

CHAPTER VII

EXAMINATION OF THE URINE

Collection of the Urine 129
The Identification of a Fluid as Urine 130
Chemical and Physical Characteristics of the Urine 132
    Color 132
    Transparency 133
    Reaction 135
    Specific Gravity 136
    Freezing-point 137
    Quantity 140
The Chemical Examination of the Pathological and Abnormal Constituents of the Urine 141
    Albumin 141
    Albumoses and Peptones 148
    Method of Freeing the Urine from Albumin 149
    Albumins Precipitated by Cold Acetic Acid 150

PAGE
The Chemical Examination of Fibrin 151
  Glucose 151
  Lactose 160
  Levulose 160
  Pentose 161
  Glycuronic Acid 162
  Acetone 163
  Diacetic Acid 164
  β-Oxybutyric Acid 165
  Indican 165
  Urobilin 167
  Biliary Pigments 168
  Blood Pigments 170
  Hæmatoporphyrin 173
  Melanin 174
  Diazo Reaction 174
  Adventitious Constituents of the Urine—Iodine, Mercury, etc. 175
Quantitative Chemical Examination of the Urine 180
  Estimation of Albumin 180
    "   of Sugar 182
    "   of Total Nitrogen 187
    "   of Urates 188
    "   of Uric Acid 193
    "   of Chlorides 199
    "   of Phosphates 199
    "   of Sulphates 201
    "   of Oxalic Acid according to *Salkowski* 202
Examination of Urinary Calculi and Concretions 206
Microscopical Examination of the Urinary Sediment 209
  Organized Sediments 224
Bacteriological Examination of the Urine 237
  Collection and Preparation of the Urine for Examination 237
  Method of Examination 238
    Bacterium coli 239
    Staphylococci and Streptococci 240
    Tubercle Bacilli 240
    Typhoid Bacilli 244
    Gonococci 244
    Proteus vulgaris 245

CHAPTER VIII

EXAMINATION OF THE URETHRAL AND PROSTATIC SECRETIONS

PAGE
Prostatic Secretion 249

CHAPTER IX

EXAMINATION OF THE BLOOD

Determination of the Specific Gravity 250
" of the Freezing-point 251
Estimation of Hæmoglobin 251
Enumeration of Blood-Corpuscles 253
Histological Examination 255
Examination of Fresh Specimens 255
" of Stained Specimens 256
Sketch of the Morphology of the Blood 259
Bacteriological Examination of the Blood 263
Examination of the Blood in Stained Smears 263
Malaria 263
Spirilla of Relapsing Fever 268
Examination of the Blood by Means of Cultural Procedures 268
Cultivation of Typhoid Bacilli 269
" of Staphylococci and Streptococci 271
Examination of the Blood by Means of Animal Inoculation 271
Serum Diagnosis 272
Macroscopic, Quantitative Agglutination Test 272
Exploratory Agglutination Test 272
Widal's Reaction 272
Pfeiffer's Test 275
The Bactericidal Test-tube Test 276
Serum Diagnosis of Syphilis According to *Wassermann* 278
Wassermann's Reaction 278

CHAPTER X

EXAMINATION OF FLUIDS OBTAINED BY PUNCTURE

PAGE
General Characteristics and Chemical Examination........ 286
Microscopical Examination........................... 290
Bacteriological Examination........................... 291
  Collection of Material for Examination............... 291
  Method of Examination.............................. 292
  The Most Important Bacteriological Findings 295

CHAPTER XI

BACTERIOLOGICAL EXAMINATION OF DISEASES OF THE SKIN

Purulent Affections of the Skin......................... 299
  Glanders.. 300
  Anthrax 301
  Tetanus.. 302
  Bacillus of *ulcus molle*............................. 304
Tuberculosis of the Skin 305
Diseases of the Skin excited by Hyphomycetes (Dermatomycosis)................................... 305
  Favus.. 309
  Trichophytosis 311
  Tinea sycosis...................................... 312
  " *circinata*................................... 313
  Pityriasis versicolor............................. 315
  Erythrasma... 316
  Spirocheta pallida.................................. 317

CHAPTER XII

THE USUAL METHODS OF BACTERIOLOGICAL EXAMINATION, FORMULÆ OF STAINS, AND CULTURE MEDIA

Examination in a Hanging-Drop 327
Examination in Stained Smears.......................... 328
  Preparation of the Specimens......................... 328

CONTENTS

PAGE

Examination in Stained Smears:

Staining Methods and Staining Solutions.............. 329

" Stock Solutions.............................. 329

" of Tubercle Bacilli and other Acid-fast Bacilli 330

" of Diphtheria Bacilli 332

" of Gonococci................................. 334

" of Spores...................................... 334

" of the Capsules of Anthrax Bacilli........... 335

" of Flagella 336

" of Fungi 337

" of Blood Specimens.......................... 338

Examination of Cut Sections............................ 339

Universal Staining Methods for Demonstrating Bacteria in Sections 341

Special Staining Methods............................... 342

Cultural Methods 344

Preparation of Culture Media......................... 344

The Cultural Methods most frequently Employed...... 356

Aerobic Cultures.................................... 356

Anaerobic Cultures 359

Determination of the Biological Characteristics of Bacteria 361

Methods of Animal Inoculation.......................... 363

INDEX... 365

LIST OF FIGURES IN THE TEXT

FIG. PAGE
1. Oidium Albicans—"Soor Fungus" 10
2. Fibrin Clots from Pneumonic Sputum................. 27
3. Spirals from the Sputum (*natural size*)............... 29
4. Spirals from the Sputum (*magnified*) 29
5. Hyaline Membrane of an Echinococcus Cyst........... 31
6. Echinococcus Hooks 31
7. Actinomyces Granules (*low power*).................... 32
8. Actinomyces Granules (*unstained specimen*) 33
9. Elastic Fibres from the Sputum 35
10. Leyden Crystals (*magnified* 300 *times*) 36
11. *Boas'* Fecal Sieve.................................. 77
12. *Schmidt's* Fermentation Apparatus 89
13. Amœba Coli 98
14. Balantidium Coli.................................. 98
15. Tænia Solium..................................... 99
16. Tænia Saginata.................................... 100
17. Scolex of Bothriocephalus Latus...................... 100
18. Oxyuris Vermicularis............................... 101
19. Ascaris Lumbricoides............................... 102
20. Tricocephalus Dispar 102
21. Anchylostoma Duodenale 103
22. *Beckmann's* Cryoscope............................... 138
23. *Einhorn's* Saccharometer............................ 157
24. *Jolles'* Azotometer................................. 191
25. Amorphous Urates.................................. 213
26. Calcium Oxalate................................... 215
27. Neutral Calcium Phosphate 216

LIST OF FIGURES IN THE TEXT

FIG. PAGE
28. Triple Phosphate 218
29. Tyrosin—Cystin—Leucin 219
30. Cystin Crystals 220
31. Hippuric Acid 221
32. Fatty Acid Needles 223
33. Epithelial Cells 224
34. Squamous Epithelial Cells 226
35. Cylindroids 232
36. Urinary Filaments 233
37. Substances Found in the Sediment of Urine 236
38. *Ehrlich's* Copper Plate 257
39. Favus Fungi 309
40. Hair in Sycosis 313
41. Epidermal Scales 314
42. Spirochetæ 317
43. Migrating Cells and Papillary Bodies 325

LIST OF COLORED PLATES

PLATE	FIG.		REFERRED TO ON PAGE
I,	A.	Diphtheria Bacilli	3
I,	B.	Diphtheria Bacilli	3
II,	C.	Sputum with Typhoid Bacilli	40
II,	D.	Pneumonic Sputum	48
III,	E.	Pneumonic Sputum	48
III,	F.	Bronchial Sputum	51
IV,	G.	Influenza Bacilli	51
V,	H.	Cholera Dejection	120
V,	I.	Uric Acid	211
VI,	J.	Ammonium Urate	213
VII,	K.	Nephritis in Pregnancy—Hematoidin Crystals	222
VIII,	L.	Casts from Icteric Urine	230
IX,	M.	Leucocytes	227
IX,	N.	Red Blood-Corpuscles	229
X,	O.	Hyaline Casts	230
X,	P.	Spermatozoa	235
XI,	Q.	Echinococcus Hooklets	235
XI,	R.	Bacterium Coli	239
XII,	S.	Tubercle Bacilli	240
XII,	T.	Gonococci	247
XIII,	U.	Macrocytes, Microcytes, etc	262
XIII,	V.	Tertian Parasite	265
XIV,	W.	Tertian Parasite	265
XIV,	X.	Chronic Tropical Malaria	267
XV,	Y.	Spirilla of Relapsing Fever	268
XV,	Z.	Anthrax Bacilli in Rabbit's Blood	301
XVI,	a.	Tetanus Bacilli	302

CHAPTER I

BACTERIOLOGICAL EXAMINATION OF THE SECRETIONS AND DEPOSITS IN THE MOUTH AND PHARYNX

In the bacteriological examination of pathological products found in the mouth and pharynx, the place of first importance belongs to the diphtheria bacilli. Of secondary importance only are the staphylo-, strepto-, pneumo-cocci, influenza bacilli, and the diplobacillus of *Friedlaender*, which appear as independent exciters of inflammation in angina, as well as producers of mixed infection in diphtheria. Finally, the "soor fungus," *Oidium albicans*, should be mentioned. In coatings on the tonsils, which are excited by the *Oidium albicans*, diphtheria bacilli are also not infrequently found.

Collection of Material to be Examined

For the collection of material from the mouth and pharynx it is best to use the small apparatus which can be obtained from any supply station. This consists of a strong test-tube containing a piece of wire, one end of which is placed in the plug which closes the tube, while the other carries a swab of common cotton. The test-tube and contents are sterilized by dry heat for half an hour at a temperature of 160° C.,[1] and then placed in a suitable box, containing directions for use and a blank to be filled out by the physician, stating duration of illness, locality from which the material to be examined is taken, etc.

---

[1] All degrees of temperature quoted in this book are Celsius.

To obtain the material for examination, the swab is brushed firmly across the suspected coating and returned immediately to the test-tube (care being taken that it touches nothing but the coating in question). No antiseptic (gargle or application) should be used for some time before the collection of the material, as the growth of the diphtheria bacillus is inhibited by even mild antiseptics.

Morphological and Staining Characteristics of Diphtheria Bacilli

Diphtheria bacilli are non-motile rods which show differences in their morphological characteristics depending upon the nature of the culture media, the age of the culture, and the temperature at which they are cultivated. They vary considerably in length, so that short, medium, and long forms may be differentiated. A six to ten hours' growth on pure serum, or *Loeffler's* blood serum, consists principally of long, partly straight, partly slightly curved bacilli, club-shaped or pointed at both ends. In older cultures the spindle, dumb-bell, and lancet forms are seen. Small, highly refractive points can be seen in the protoplasm of the long forms. The formation in colonies of the diphtheria bacilli, of loosely arranged groups, in which the individual bacilli lie crosswise over each other, is characteristic. Especially in *Klatsch*-preparata from young serum cultures and in membranes in which the diphtheria bacilli are present in great numbers, a picture is seen, which may be imitated by holding the outspread fingers of one hand, in various combinations, over or beside those of the other (*Neisser*).

Diphtheria bacilli stain easily with dilute aniline dyes. Dilute *Ziehl's* solution (1:9), and *Loeffler's* methylene blue, are especially adaptable. The former stains in one minute, the latter in two minutes, without heating. Diphtheria bacilli stain according to *Gram*. Bacilli from young cultures stain evenly, while in smears from older cultures they usually show one or more unstained spots. Bacilli stained with *Loeffler's* methylene blue, as a rule, show either at one or both ends, granules more deeply stained than the rest of the protoplasm. These polar granules (*Babes-Ernst's* bodies) are especially distinct in specimens stained according to the *Roux* or *Neisser* methods (Plate I, Fig. A). *Roux's* solution (cf. p. 333) is made by mixing 1 part dahlia-violet solution with 2 parts methyl-green solution. The mixture keeps well and produces no precipitate. It stains in two minutes without heating. *Neisser's* is a double staining method (formula cf. p. 333).

1. *Old Method.*—Stain with acetic acid methylene blue twenty seconds, wash with distilled water, counterstain with Bismarck brown ten seconds, wash, etc.

2. *New Method.*—Stain with Solution I (cf. formulæ of stains) for about thirty seconds, wash with distilled water, counterstain with Solution II, also for about thirty seconds. The bacilli then appear brown, the oval polar granules dark blue (Plate I, Fig. B). As a rule, each bacillus shows two granules, one at each end. Some, however, have but one, at one end, while still others have a third in the middle. It is common to find two bacilli at an obtuse angle to each other, having together three or four granules. This method of staining is only sure of success when the smears are made from serum cultures, which are at least nine and not more than twenty to twenty-four hours old, and have been grown at a temperature of 34° to 37° C. It is further important that the smears be very thin. It is necessary to test newly made solutions for *Neisser's* method, as the staining power of

different solutions varies. *Neisser's* method is of value in differentiating between diphtheria bacilli and other bacilli resembling them.

Cultural Characteristics

For the cultivation of diphtheria bacilli a temperature of at least 20° C. is necessary. They grow best between 33° and 37° C. They flourish on all the usual culture media which have a slight or distinct alkaline reaction. For diagnostic purposes bouillon, glycerine agar (5 to 7 per cent.), and especially blood serum, are used.

After one to two days' growth, bouillon is either evenly clouded, with flakes in it, or a fine granular precipitate has developed, which collects on the sides and bottom of the glass. Not infrequently a thin, granular, and easily destroyed film forms on the surface of the bouillon. The bouillon, originally faintly alkaline, becomes acid after forty-eight hours' growth of diphtheria bacilli in it. The growth on agar is scanty, but that on glycerine agar is richer. The surface colonies appear transparent and grayish-white; examined with low power they show a characteristic granular surface and an irregular delicate border. The most luxuriant and quickest growth of diphtheria bacilli is that on blood serum. For their cultivation from membranes it is especially suitable, as it has a selective action against the other bacteria of the mouth. On blood serum the diphtheria bacilli grow first, while other organisms, which may at the same time be present, develop later.

Either pure blood serum, which may come from cattle, sheep, or horses is used, or the so-called *Loeffler's* serum, which is a mixture of 3 parts calf or sheep serum with 1 part of 1 per cent. grape-sugar bouillon (cf. p. 354).

On solidified serum the diphtheria bacilli have often, after but six hours' growth, developed very small transparent colonies. After twenty-four hours' growth the colonies are about the size of a pin's head, round, prominent, and yellowish-white. If the colonies lying close together coalesce, a yellowish-white coating is formed, which still presents a distinctly granular appearance.

Animal Inoculation

Inoculation of guinea-pigs is used for diagnostic purposes as a means of differentiating the diphtheria bacilli from bacteria resembling them. Guinea-pigs weighing 200 to 300 grammes are inoculated subcutaneously with 2 cc of a forty-eight hours' bouillon culture. The animals become sick quickly. After twelve to twenty-four hours a distinct infiltration can be felt at the point of inoculation; after two to three days the animal dies. The post mortem shows at the site of inoculation a jellylike, œdematous, blood-stained swelling of the subcutaneous tissue. The peritoneal and pleural cavities contain serous and frequently hemorrhagic exudates. The condition of the adrenal bodies is characteristic. They are enlarged and hyperæmic, and their tissue is permeated by small punctiform hemorrhages. In the œdema fluid at the site of inoculation diphtheria bacilli can usually be detected by cultural methods.

If the bacilli were less virulent, the animal dies later, and the post-mortem appearances are not so typical. If still less virulent, merely a local inflammation at the site of inoculation is produced, which causes necrosis of the skin and eventually heals.

Differential Diagnosis

In the differential diagnosis pseudo-diphtheria bacilli (*Hoffmann*) and xerosis bacilli come into consideration.

The former are among the normal inhabitants of the mouth, the latter are found on the conjunctiva and in the nose. The morphological differences between pseudo- and true diphtheria bacilli are most evident in *Klatsch*-preparata from young (six to ten hours') blood-serum cultures. Here the pseudo-diphtheria bacilli appear usually as short, plump, often wedge-shaped rods; the characteristic long forms, which diphtheria cultures of the same age show, are missing. The typical grouping of the bacilli is also lacking. Pseudo-diphtheria bacilli lie usually with the long sides parallel, side by side, arranged like palisades. In smears from older (sixteen to twenty-four hours') cultures the differentiation may be difficult.

Pseudo-diphtheria bacilli differ from true diphtheria bacilli, in their staining properties, by their failure to stain according to *Neisser's* method. Although sometimes pseudo-diphtheria bacilli, if stained by this method show pole kernels, they are always found but scarcely, never as regularly as in diphtheria bacilli. It is best to use cultures of from twelve to twenty-four hours for the differential diagnostical staining, because the absence of the pole kernels in cultures of from six to twelve hours means just as little as the presence in older colonies.

In their cultural characteristics they differ by their luxuriant growth on agar and, at first, slower development on serum. The colonies are grayish-white, moistly glistening, and somewhat waxy in appearance. It is remarkable what soft and melting consistency they show, when touched with the platinum needle in contradistinction to the more rigid diphtheria bacillus colonies.

The faculty of diphtheria bacilli of forming acid in culture media containing sugar may also be of differential diagnostical value.

Diphtheria bacilli decompose dextrose and levulose under acid formation, pseudo-diphtheria bacilli, as proven in numerous types of various origin are almost always inactive in both kinds of sugar, and, but in rare cases, they are active in one, never in both.

The following culture media are used for testing acid formation: Three parts of ox serum, which is rendered sterile or made germ free by discontinuing sterilization at 55°, are added to one part of sterile bouillon, which is free of sugar. To each 90 parts of this mixture are added 10 parts of a litmus solution, which contains 10 per cent. dextrose, resp. levulose, in order to obtain 2 media, of which one contains 1 per cent. dextrose, the other 1 per cent. levulose. The litmus sugar solution before being added, has to be sterilized on two consecutive days, for five minutes each day. The culture media are then filled in test-tubes and are kept on three to five consecutive days, for two hours each day, in the incubator at 55°.

Procedure.—A test-tube of levulose and a test-tube of dextrose are each inoculated with a loop of the pure culture to be examined. After twenty-four hours in the incubator at 37° the litmus solution appears red in the test-tubes, which have been inoculated with diphtheria bacilli and the serum albumin is precipitated.

The test-tubes inoculated with pseudo-diphtheria bacilli as a rule appear unchanged, only sometimes a little acid formation can be traced.

The same result is also obtained from nutrose litmus bouillon:

Liebig's meat extract,		
Pepton	āā	2.0
Aq. dest		150.0

Boil in a steam-pot until the pepton is dissolved, then neutralize with a 10 per cent. soda solution, again boil up and filter. Then are added a solution of 2.0 nutrose in 50 cc water, 2.0 dextrose or levulose and 20 cc litmus solution (*Kahlbaum*). After precipitation in sterile test-tubes the culture medium is again sterilized by steam on three consecutive days, fifteen minutes each time.

Xerosis bacilli resemble in appearance the long forms of the diphtheria bacilli, but produce a slower growth on serum and glycerine agar. After six hours' growth on blood serum they are so slightly developed, and cling so tightly to the culture media, that in *Klatsch*-preparata no typical groups appear. Even after twenty hours the development of colonies is not marked, and still allows the making of *Klatsch*-preparata, which, with true diphtheria bacilli, is no longer possible because of their luxuriant growth.

It is not always possible to distinguish xerosis bacilli from true diphtheria bacilli by *Neisser's* method, as xerosis bacilli also frequently show polar staining. In relation to acid formation in culture media of dextrose and levulose they react like pseudo-diphtheria bacilli. Animal inoculation is the surest means of differentiation, as xerosis bacilli show themselves non-virulent.

Order of Examination

Four unused cover glasses, sterilized in the flame, are smeared with the material to be examined. The smears are stained with ten times diluted *Ziehl's* solution, with *Loeffler's* methylene blue, according to *Roux* and according to *Gram*. Cultures are also planted upon blood serum and glycerine agar.

Examination of the Smears.—In some cases, even in the smears, numerous bacilli are seen which, both in form and position, resemble diphtheria bacilli, so that from this fact alone a probable diagnosis of diphtheria may be made: the result of the cultures must, however, be awaited before giving a definite report. In a very large number of cases, however, only a few isolated suspicious-looking bacilli are found, or often these are missing. Diplo-, strepto-, staphylococci, bacilli which do not stain by *Gram*, spirilli, etc., are present. Nevertheless, the case may be one of diphtheria, as the result of the cultures will later show.

Examination of the Plates.—*Klatsch*-preparata are made from the serum plates after they have been six hours in the incubator, and are stained with fuchsin or *Loeffler's* methylene blue. If the above described bacilli arranged in typical groups are found, the diagnosis of diphtheria is very probable. After ten to eighteen hours' growth the plates are again tested. If the material under examination contained diphtheria bacilli capable of development, they will by this time have developed the characteristic colonies in nearly pure culture. Smears are now made from the serum plates and stained with *Loeffler's* methylene blue, and according to *Roux* and *Neisser*. If these contain almost exclusively bacilli which show the characteristic polar staining, the diagnosis of diphtheria can be made, *provided that the material examined came from a sick person and was taken from the pharynx.*

If after twelve to twenty-four hours cocci alone have grown, it is very improbable that the case is one of diphtheria; however, the plates must be examined again on the following day, as in rare cases diphtheria bacilli develop late—namely, when gargles, etc., have been used shortly before obtaining the material for examination.

Colonies of diphtheria bacilli can also be seen on glycerine agar plates after twelve hours' growth at 37° C.; but the other micro-organisms which were present in the material have also developed by this time. These bacteria are identified according to the methods described under Examination of Sputum.

Oidium Albicans—"Soor Fungus" (Fig. 1)

The detection of the "soor fungus" succeeds best in unstained specimens, made by teasing a particle taken from the suspected coating in a drop of water or glycerine. In such specimens are seen double-contoured hyaline hyphæ (mycelia), having transverse septa and indentations, and often having lateral branches which interlace with one another. Among the hyphæ (mycelia) appear the conidia, sometimes spherical, sometimes cylindrical.

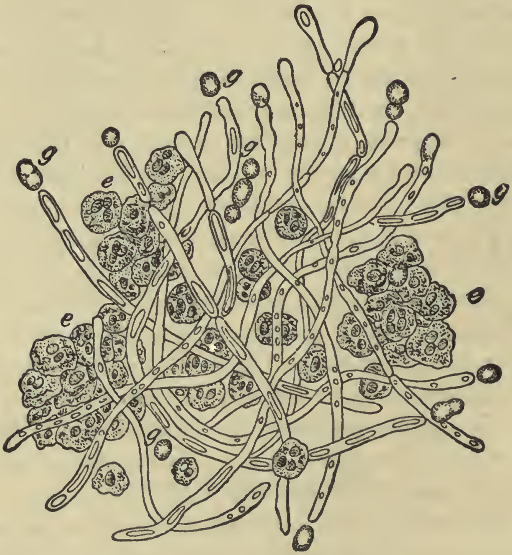

FIG. 1.—Oidium Albicans.

The specimens may be stained according to *Gram* or *Kuehne-Weigert* (cf. p. 338).

The cultivation of the fungus is unnecessary for diagnosis. It can, however, be easily grown on all the usual culture media; on agar it produces porcelainlike, glistening white colonies, composed of yeastlike oval cells, and containing only isolated, short hyphæ (mycelia). These develop more richly in gelatine stab cultures, in the lateral branches extending from the stab canal. Gelatine is not liquefied.

In the coatings of the tonsils which are produced by the oidium albicans, are found occasionally at the same time diphtheria bacilli, when a culture is made.

Angina Vincenti (s. Plautii)

The diagnosis of the *Plaut-Vincent* angina is made from the stained smear. But owing to the fact that this disease is sometimes combined with diphtheria, the material must always be inoculated in blood serum.

The specimens are stained with a diluted *Ziehl's* solution (cf. solutions for staining) or according to *Giemsa.*

The stained specimens show that they are composed of coating, which is easily removable, a paste of a grayish-brown or slightly greenish color and ill-smelling, necrotic tissue, which contains a very large quantity of fusiform bacilli and mostly numerous spirochetæ. In the diphtheroid or pseudo-membranous form of the angina Vincenti as a rule only fusiform bacilli are found, in the ulcerous type are also found spirochetæ. In fresh cases the bacilli fusiformes and the spirochetæ appear almost in pure cultures, whereby only a few of the ordinary mouth bacteria are found. Only at about the termination of the disease the accompanying bacteria are brought more into the foreground.

The bacilli fusiformes are mostly long, slender rods, pointed at their ends, have a slight swelling in the centre, and therefore appear fusiform; they are straight or slightly curved (comma-shaped). In the stained specimen we see in the centre an oval, unstained vacuole. Besides these typical, long, slender forms are also found shorter rods and thin, long, threadlike bodies, pointed at their ends, frequently **S**-shaped. In the smears the bacilli fusiformes are found mostly singly spread over the entire visual field, often in twos, which form more or less obtuse angles; more seldom in heaps, in which case their formation resembles the typical form of diphtheria bacilli.

According to *Gram's* method they are negative.

Regarding their motility the views are divided. Some authors believe them to be immovable, others say that they move but slowly, *Graupner* could see active motility, although quickly diminishing.

We can culture the bacilli fusiformes only anaerobic in serum or ascites agar at 37°. After twenty-four to forty-eight hours we can see fine yellowish-white colonies, which have a somewhat darker centre with radiations in all directions. The cultures have a fetid odor.

The spirochetæ which in most of the cases accompany the bacilli fusiformes, look the same as the spirochetæ which we find ordinarily in the oral cavity, especially in the dental deposit (according to *Muehlens*, spirocheta buccalis and the middle form of the mouth spirochetæ.` They are corkscrew-shaped, very motile in shape and size, however, very much different from each other. We find together thin and thick spirochetæ, some which have three to five windings, and longer ones with ten and more windings. Most of them are flat and irregular; they straighten out only while in motion. The windings of others again show resemblance to the spirocheta pallida.

(Examination of the fresh specimen with the diaphragm shut.)

The spirochetæ do not stain so readily as the bacilli fusiformes; they are also negative according to *Gram's* method. They are found in the smears, isolated or in more or less thick heaps and entwined in each other, like in nests.

The culture of the mouth spirochetæ can be obtained in pure cultures under anaerobic conditions in serum agar (*Muehlens*). Not before eight to ten days appear very fine colonies at the bottom, which render the culture media cloudy. The cultures have a fetid odor.

Stomatitis Ulcerosa.—The specimens which are stained from the coverings of the ulcers are identical with those in angina Vincenti.

Meningococci.—The meningococci are found in the mucus of the naso-pharyngeal cavity in patients suffering from epidemic cerebro-spinal meningitis, especially in the beginning of the disease and in persons who have come in contact with such patients.

The specimen is to be taken from the upper part of the naso-pharyngeal cavity around the pharynx tonsil by way of a right-angularly-upward-bent probe, which is put in the mouth and then upward behind the soft palate.

Morphological and Tinctorial Properties.—The meningococci are diplococci which resemble gonococci in shape and adjustment. The frequent presence of tetrades is characteristic for specimens and cultures. The individual cocci often differ in size. Sometimes very large specimens are found, besides normal and well-stained cocci, and often cocci three times the size smaller and poorly stained. This shows especially in older cultures after about forty-eight hours. In the secretions sometimes the meningococci are found grouped in small heaps within the pus cells.

According to *Gram's* method they are negative, the same as the gonococci and the micrococcus catarrhalis.

Cultural Properties.—The meningococci develop best in ascites agar (one part ascites fluid plus three parts agar) at 37°. They also grow very well in *Loeffler's* blood serum (cf. culture media).

The meningococci do not grow in ordinary agar in the first generation, but they get accustomed to this medium, especially when frequently transplanted in later generations. After twenty-four hours' growth in ascites agar colonies are formed of 2 to 4 mm, which have a grayish-white appearance, but show a lustre of mother-of-pearl by transmitted light. Under the microscope they are grayish-yellow, smooth-edged, homogeneous or indistinctly granulated. Two distinct zones are in older colonies, a slightly elevated, central zone, and a flat, peripheral. On the sloping surface of ascites agar a homogeneous, grayish colony is formed, ascites bouillon becomes cloudy, and sometimes a top skin is formed. The meningococci do not coagulate milk nor do they grow much in it. They transform dextrose and maltose into acids, but they cannot attack levulose, mannit, milk-sugar, cane-sugar, dulcit, galactose and inulin. *v. Lingelsheim* prepared an ascites litmus sugar agar (cf. culture media) to ascertain this reaction of the meningococci. The medium containing maltose or dextrose is stained red through the growth of the meningococci, if the other kinds of sugar (levulose, etc.) are added, the medium remains blue. For diagnosis the test with maltose, dextrose, and levulose is sufficient. The cultures are not very resistible. They must be kept at 37°, and they must be transplanted in a new medium—at first daily, later every six to seven days.

The *animal test* is of no diagnostic value.

The *agglutination test.* By immunizing rabbits, and especially horses with meningococci, a serum is obtained which agglutinates most of the species, which have the characteristic properties of the meningococci, but some of them are not so easily agglutinable, and, therefore, they cannot be differentiated by the agglutination test. It is best to take cultures of forty-eight hours' growth. The inoculated test-tubes must be kept standing for twenty-four hours at 55°, covered with a rubber cap, before ascertaining the result.

Differential Diagnosis.—The differential diagnosis is to be made against the gonococcus and a number of *Gram* negative diplococci, which are found in inflammatory affections of the bronchi and also in the normal mucus of the pharynx (micrococcus catarrhalis, diplococcus flavus species, micrococcus pharyngis cinereus).

It is impossible to make a distinction between these diplococci in the stained specimen, only by their cultural properties and by the agglutination test. The latter does not hold good in gonococci, because the gonococci are agglutinated from a very much diluted meningococcus serum. The other diplococci are influenced by it in no higher dilution than from normal rabbit or horse serum. Sometimes they are agglutinated spontaneously in salt solutions. A sure differential diagnosis between meningococci and gonococci can be made from cultures only.

The colonies of the gonococci are smaller than those of the meningococci, they do not coalesce; they are clammy and slimy, and generally distinctly granulated at a low magnification. Gonococci only grow in media, which contain human albumin in a non-coagulated state; meningococci develop also in *Loeffler's* blood serum.

The *micrococcus catarrhalis* also differs from the meningococci by its cultures; besides that it grows already in the first generation in ordinary agar and is unable to attack any of the above-mentioned kinds of sugar.

The cultures of the species *diplococcus flavus* (according to *v. Lingelsheim* there are three species) are characterized by the formation of a yellow pigment.

The *micrococcus cinereus* differs from the meningococcus microscopically by its coarse, irregular, occasionally oblong texture (*v. Lingelsheim*). Its colonies look like those of the micrococcus catarrhalis, and it is likewise unable to attack any of the above-mentioned kinds of sugar.

The examination for meningococci must be made as soon as possible after the mucus has been removed from the pharynx. The diagnosis of the presence of meningococci in the pharyngeal secretion can only be established by making cultures because of the presence of the above named diplococci, which look alike microscopically. The probe to which the mucus adheres is smeared over three ascites plates. After twenty-four hours' growth at 37° the suspicious-looking colonies are stabbed and transported to sloping ascites agar. The cultivated pure cultures are identified by the agglutination test and by their cultural properties.

CHAPTER II

BACTERIOLOGICAL EXAMINATION OF NASAL SECRETIONS

The nasal secretion is removed for examination by means of a swab or platinum wire, with the aid of a head mirror.

The material is examined in stained smears, by cultural methods, and by animal inoculation. The bacilli which come especially into consideration are diphtheria bacilli, tubercle bacilli, lepra bacilli, influenza bacilli, and the micro-organisms belonging to the group of the diplobacillus of *Friedlaender*—the so-called ozæna and rhinoscleroma bacilli.

Diphtheria Bacilli.—The recognition of diphtheria bacilli is accomplished in the same manner as in the examination of pharyngeal coatings. However, animal inoculation is necessary for the verification of the cultivated bacteria in cases in which the diphtheria has not spread from the pharynx to the nasal cavity, because of the especially frequent presence in the nose of bacteria resembling diphtheria bacilli.

Tubercle Bacilli.—Tubercle bacilli are detected by means of stained smears. For the differential diagnosis between tubercle bacilli and other acid-fast bacilli which may be normally present in the nose, animal inoculation must be used. Lepra bacilli can often be distinguished from tubercle bacilli by their characteristic position and arrangement.

Lepra Bacilli.—Smears are made from the secretion removed from the nose with a platinum wire, and stained according to the method for tubercle bacilli and according to *Baumgarten* (cf. p. 332 and Examination of Sputum). Bacteria belonging to the group of the diplobacillus of *Friedlaender* are very frequently present in the nose of a healthy person. The micro-organisms which have been detected in ozæna and rhinoscleroma cannot, with certainty, be separated from the diplobacillus of *Friedlaender*. In their morphological and cultural characteristics, as well as in their behavior when inoculated into animals, they agree almost exactly or exactly with it; such variations from the typical as they do occasionally show, are not more marked than those which may be seen in different cultures of the diplobacillus of *Friedlaender* itself. As yet the attempt to differentiate between the three kinds of bacteria by means of agglutination tests has failed (*Klemperer* and *Scheyer*). Concerning the detection of these bacteria and influenza bacilli, cf. Examination of Sputum, p. 22.

CHAPTER III

BACTERIOLOGICAL EXAMINATION OF CONJUNCTIVAL SECRETION

Material for examination may be obtained by means of the swabs suggested for obtaining material from the throat. If the secretion is very thin, sterile capillary tubes are used, which, after the material is obtained, are sealed at both ends by melting in the flame. If the material is to be examined at the bedside, it is best obtained with a sterile platinum wire.

The examination is usually made by means of stained smears and cultural methods. Animal inoculation is resorted to only when diphtheria or tubercle bacilli are suspected.

Diphtheria Bacilli.—Detection is accomplished according to the method described on p. 8. In the differential diagnosis xerosis bacilli must be borne in mind (cf. p. 8).

Tubercle Bacilli.—In tuberculosis of the conjunctiva tubercle bacilli may occasionally be detected even in the smears; in many cases, however, animal inoculation is necessary. As material for inoculation, the secretion from an ulcer, or a small piece excised from the conjunctiva, may be used.

Often in these cases the injection is made into the anterior chamber of the eye of a rabbit. The animal being fastened on the operating board and the eye anæsthetized with 10 per cent. cocain, a fold of conjunctiva is grasped with the thumb forceps and the eyeball drawn downward and incised close to the upper edge of the cornea. To prevent injury to the iris, the lancet must be introduced parallel to it, and withdrawn with its point directed toward the cornea (by sinking the handle). The material to be inoculated is introduced into the anterior chamber of the eye through the wound on the upper border of the cornea, by means of a syringe or iris forceps. To guard against prolapse of the iris, a solution of eserin is dropped into the eye immediately. During the next few days a solution of atropin and cocain should be dropped into the eye to allay the irritation following the operation.

Depending upon the number of tubercle bacilli introduced, and upon whether from the start they lay free, or were embedded in the tissue, there develops, after one or more weeks, a tuberculosis of the iris, which may finally lead to a caseous phthisis of the eyeball. If the tuberculosis spreads farther, it attacks first the neighboring lymph-nodes and then the lungs, and eventually produces a general tuberculosis, which, after weeks or sometimes months, causes the death of the animal.

Gonococci.—The detection of gonococci is made by means of smears stained with dilute methylene blue and by *Gram's* method. For their identification cultures must be made, as other diplococci are found in the conjunctival secretion which resemble them both morphologically and in their staining characteristics (cf. Examination of Urethral Secretion).

Bacilli of Koch and Weeks.—These are found as the exciters of both acute and chronic conjunctivitis in the secretion of the conjunctival sac. In smears made from this secretion and stained with dilute borax methylene blue, especially during the rise and height of the conjunctivitis, though also in chronic cases, numerous fine slim bacilli of varying length, resembling influenza bacilli, are found. These lie either within the pus cells, which appear stuffed with them, or outside of them. They are decolorized by *Gram.*

The bacilli cannot, as a rule, be cultivated upon plain agar. They flourish, however, on human blood and ascites agar, and develop, after twenty-four to forty-eight hours, small moist colonies resembling dew-drops.

Diplobacillus of Morax and Axenfeld.—Conjunctivitis excited by these diplobacilli produces frequently but little secretion. To prepare the smears, the mucus is used, which is, though in small amount, usually present on the caruncle. In smears stained with dilute methylene blue the bacilli, which in appearance resemble the diplobacilli of *Friedlaender*, are seen, some within, some free, and some lying upon the epithelial cells. They are usually arranged in twos, and present themselves as plump bacilli, resembling a rectangle with blunted corners. They are decolorized by *Gram.*

The diplobacilli grow on blood serum or on serum agar. Blood serum is liquefied, and after twenty-four hours the surface appears uneven, due to the presence of small, moist, somewhat sunken and translucent spots, which gradually become deeper and deeper (*Axenfeld*). In pure cultures involution forms develop—partly grotesque, partly very large—even after but two days' growth.

Influenza bacilli, pneumo-, strepto-, staphylo-, and meningococci are also found in the conjunctival secretion as exciters of conjunctival catarrh. Concerning the detection of these bacteria, compare Examination of Sputum and Examination of Fluids Obtained by Puncture.

CHAPTER IV

EXAMINATION OF THE SPUTUM

Method of Obtaining Material for Examination

The sputum must be collected in a clean vessel. It is best that the vessel be sterile, and that the sputum be examined as soon as expectorated. When this is not possible, it is well to collect the sputum in a 1 to 2 per cent. carbolic acid solution, which has been proved to be sufficient to check further bacterial development. More concentrated carbolic acid solutions are to be avoided, as they render the sputum unfit for examination. Such sputum cannot, of course, be used for cultural tests.

The patient should be instructed to bring only such sputum for examination as has been really raised by coughing, and not by hawking. To prevent contamination, the mouth should be rinsed several times with freshly boiled water before expectoration. When the expectoration is slight, it is best to examine the morning sputum, or when it is a question of determining the presence of tubercle bacilli, to collect the expectoration of the entire day in a vessel which can be tightly closed, and which contains 1 to 2 per cent. carbolic solution. To excite expectoration potassium iodide may be administered, or moist compresses may be bound over both shoulders during the night, followed in the morning by a cold rub down. The coughing excited by the shock will expel the excretion which has collected under the influence of the moist warmth.

General Characteristics

The macroscopical examination of the sputum, which should always precede the microscopical, discloses its general characteristics. For this purpose the sputum is poured into a flat glass dish, the so-called Petri dish, and examined over a dark background. Notice should be taken of the quantity, odor, stratification, color, and consistency of the sputum, and any especially prominent ingredients.

Quantity of Sputum.—Though this varies exceedingly in the majority of the diseases of the respiratory tract, yet for certain of them the large amount of sputum produced is in itself characteristic. For example, a noticeably large quantity is expectorated in cases of empyema, which has ruptured into the lungs, and in bronchiectasis, pulmonary gangrene, and abscess.

Odor.—Freshly expectorated sputum has usually no characteristic odor. It is foul-smelling only when it has decomposed as the result of long standing. Sputum in diseases in which its decomposition has taken place within the body has even at the time of its expectoration a repugnant, often a cadaverous foul odor (pulmonary gangrene, putrid bronchitis, etc.).

Stratification.—In bronchiectasis, putrid bronchitis, and pulmonary gangrene, the sputum separates shortly after its expectoration into three strata: an upper foamy stratum, greenish-yellow in color; a middle translucent, serous stratum, and a lower non-transparent stratum, purulent in character. Sputum in cases of lung abscess shows, usually after standing some time, two strata: an upper serous stratum, representing the pus serum, and a lower non-transparent yellow one, containing the cellular elements.

Color.—The color depends generally upon the richness of the sputum in cellular elements. It is light and glassy in sputum containing few cells, non-transparent or yellow in that containing many. The color caused by the presence of blood is very striking. It is bright red and foamy in hemorrhages from eroded bloodvessels; its rusty color in pneumonia is pathognomonic; it is dark, nearly black in cases of hemorrhagic infarct and at the termination of pthisical hemorrhage. The thin liquid sputum in œdema of the lungs is, in proportion to the amount of blood contained, yellow, rose-colored, or dark red; in inflammatory œdema complicating croupous pneumonia, it resembles prune juice. The expectoration in hemoptysis resulting from neoplasms may resemble currant jelly. When sputum containing blood has decomposed, as in pulmonary gangrene, it has a brown or dirty green color. Sputum may be stained green by pigment produced by bacteria (*B. pyocyaneus*, *B. fluorescens*, *sarcinæ*, etc.).

Composition of Sputum

A distinction is made between mucous, muco-purulent, purulent, and bloody sputa.

I. **Mucoid Sputum.**—This may be pure mucoid or watery mucoid. The pure mucoid sputum is translucent, grayish-white in color, tough and thready in consistency. The watery mucoid sputum is more liquid, less tough than the pure mucoid, and frequently so rich in air-bubbles that the entire expectoration is covered with foam. The mucoid portions lie as flakes or balls in the deeper liquid substance.

II. **Muco-Purulent Sputum.**—This may be muco-purulent and nearly homogeneous, or purulo-mucoid and non-homogeneous. In the first case, the sputum forms a practically homogeneous non-transparent mass of yellowish-white appearance and of still comparatively tenacious, gluey consistency. Only by examination in direct light can the translucent mucoid portions be clearly distinguished from the purulent. The latter permeate in streaks the mucoid mass. The fine mixture of pus and mucus points to the fact that both were produced in the same portion of the respiratory tract.

In the purulo-mucoid, non-homogeneous sputum the purulent outweigh the mucoid ingredients. The purulent, greenish-yellow, non-transparent portions are not mixed with the mucous, but build either round nummular discs (sputum rotundum), or, after longer standing, flow together, sink to the bottom, and produce stratified sputum.

III. ***Purulent Sputum.***—This is greenish-yellow in color, homogeneous in appearance, and thickly liquid in consistency. Its characteristic division into strata has been mentioned.

IV. ***Bloody Sputum.***—1. Pure bloody sputum (hemoptysis is either liquid, bright red and foamy, or has coagulated before its expectoration, forming thick clumps. The question of the source of the blood is occasionally difficult to answer; from the appearance of the blood alone, differential diagnosis between hemoptysis and hematemesis cannot be made. Blood coming from the stomach has, to be sure, frequently a characteristic dark brown chocolate-colored appearance; it may, however, appear bright red and arterial. Even the admixture of stomach contents is not always conclusive, as vomiting may be caused by severe hemoptysis. The composition of the clot may, however, be of aid. In hemoptysis the blood usually coagulates more quickly and more thoroughly than in hematemesis, and the clot shows on cross-section numerous pores, caused by the admixture of air, which give it a spongy appearance. The detection of sputum flecks in the blood is decisive for the diagnosis of hemoptysis. In a great number of cases, however, only the history and examination of the patient can establish the source of hemorrhage.

2. Blood-stained sputum. The blood permeates in the form of flecks or streaks, the mucoid, muco-purulent, or purulent sputum.

3. Sputum thoroughly mixed with blood. The appearance differs according to the quality of the blood contained.

(*a*) Mucoid bloody sputum is tenacious and has a yellow to rusty brown color. It is characteristic of the alveolæ and smallest bronchi.

(*b*) Serous bloody sputum is thinly liquid, contains numerous air-bubbles and has a dark brown to black color. It is designated as prune-juice expectoration.

(*c*) Purulo-sanguineous sputum (that containing pus and blood thoroughly mixed) points to the existence of large cavities, in which it is produced by the mixture of purulent secretion, with more or less altered blood ingredients. Two forms of this sputum are recognized, depending upon whether it is expectorated soon after its secretion or after a longer retention in the cavity. In the first instance, airless, nummular clumps, with dirty red centres and distinctly red-stained periphery, are expectorated, which sink quickly to the bottom in water (*Sp. globosum fundum petens*). In the latter instance, the sputum has a homogeneous appearance and a dirty red to muddy brown color (pulmonary gangrene, bronchiectasis).

Especially Prominent Ingredients of the Sputum

The so-called kernels (*Corpuscula oryzoidea*) are pinhead to sago-sized opaque objects, yellowish-white in color and cheesy in consistency. They can be easily isolated from the purulo-mucoid sputum in which they are usually found. They have their origin in cavities, and are of diagnostic worth, as they are usually very rich in tubercle bacilli and elastic fibres. These kernels should not be confused with tonsillar plugs and pieces of food which may resemble them. They are distinguished from these by microscopical examination.

Dittrich's plugs are grayish-white particles, sometimes as large as a bean, which are found in the sediment of sputum in pulmonary gangrene.

Portions of tissue may be present in sputum in ulcerative processes of the respiratory organs. They are most frequently present in the sediment of gangrenous sputum, and appear as black or dark gray villous shreds, which, when examined microscopically, are seen to be necrotic lung tissue. Pieces of tumors may be present in cases of neoplasm of the lungs; their presence is, however, extremely rare.

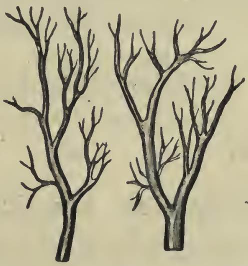

FIG. 2.—Fibrin Clots from Pneumonic Sputum. (After *v. Jaksch.*)

Curschmann's spirals appear as spiral threads sharply defined against the background of structureless sputum. They are grayish-white in color and noticeably firm in consistency (Figs. 3 and 4).

Fibrin clots (Fig. 2) are white, cylindrical, branching structures which may be several centimetres in length. They result from clotting of fibrin in the bronchi, of which they are casts. They must not be confused with the clots of thickened mucus which macroscopically resemble them, and which are much more common in sputum than true fibrin clots. They can be distinguished from one another by microscopical and microchemical examination. The branchings can be more clearly seen after the clot has been washed in water.

Actinomyces kernels appear, principally, as comparatively solid bodies the size of grains of sand and yellowish-green or black in color. There are in addition, however, gray, easily crushed kernels which appear gelatinous and glassy, resembling clumps of mucus.

Food particles are often mixed with sputum, and are present especially in the mucous portions coming from the upper air-passages.

Microscopical Examination

The particles which are to be examined microscopically are best isolated from the surrounding sputum by the aid of two platinum wires, which can be sterilized in a flame before and after using, smeared on a slide, covered with a cover glass, and examined first with the low, then with the high, power.

In the examination of fresh smears, microchemical reactions are often used. The reagents most frequently used are dilute acetic acid, and an 8 to 10 per cent. potassium hydrate solution. To obtain thorough admixture of the reagents with the material to be examined, they are rubbed together on the slide before being covered with the cover glass.

The objects which have attracted attention during the macroscopical examination of the sputum over a dark background are the first to be examined.

The **Curschmann Spirals** (Figs. 3, 4), which, because of their tough consistency, can only be crushed with difficulty between cover glass and slide, permit when held against the light, even macroscopically, a distinct spiral form to be seen. In the microscopical picture they present themselves as translucent spirals composed of numerous closely placed and delicate convolutions, in whose

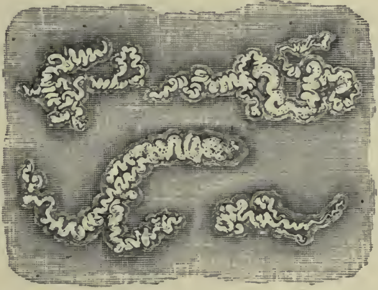

FIG. 3.—Spirals from the Sputum (Natural Size). (After *v. Jaksch.*)

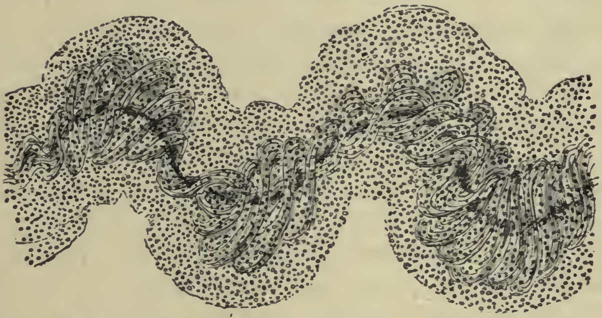

FIG. 4.—Spirals from the Sputum (Magnified). (After *v. Jaksch.*)

axis there usually runs a central thread. They are, as a rule, thickly covered with leucocytes, among which *Charcot-Leyden* crystals are often present. Usually the structure of the spiral becomes distinct only after the addition of acetic acid.

The **Fibrin Coagula** are composed of bundles of parallel refractive threads, between which more or less numerous leucocytes, as well as erythrocytes, and occasionally *Charcot-Leyden* crystals, are visible. The mucus coagula, which resemble them microscopically, are, however, composed of a homogeneous basic substance, in which leucocytes are embedded. Upon the addition of acetic acid the fibrinous structures become clearer, while the mucus coagula become cloudy, and, at the same time, their basic substance assumes a striated appearance.

The **Tissue Shreds,** which attract attention in gangrenous sputum, contain connective-tissue fibres whose alveolar arrangement identifies them as the remains of necrotic lung-tissue. Elastic-tissue fibres are rarely recognizable in them. The connective-tissue fibres are usually surrounded by a large mass composed of various kinds of bacteria, fatty detritus, fatty acid needles, triple phosphate crystals, and dark pigment granules. The parenchymatous shreds, which are present in the sputum of subacute or chronic lung abscess, contain nearly always, on the contrary, elastic fibres, either singly or in alveolar arrangement, and in addition are composed of numerous bacteria, fatty degenerated cells, and fatty acid needles, containing also, occasionally, crystals of hematoidin and cholesterin crystals, which latter are otherwise rarely present in the sputum.

Dittrich's Plugs consist principally of masses of detritus, and of an exceptional number of different microorganisms.

Portions of the Echinococcus (Figs. 5 and 6) are expectorated when echinococci are located in the lungs, or when

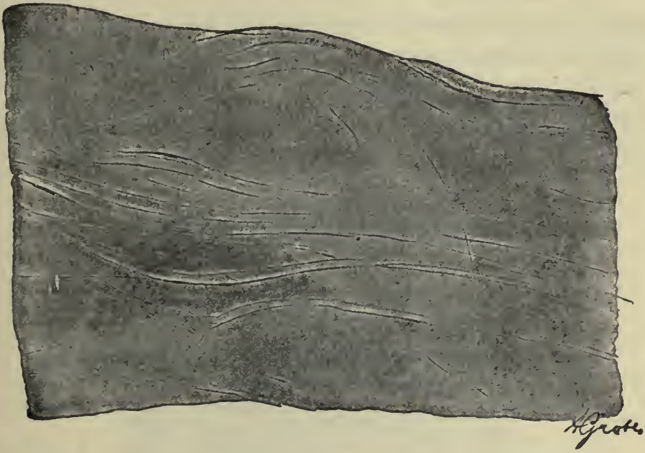

FIG. 5.—Hyaline Membrane of an Echinococcus Cyst. (After *v. Hansemann.*)

an echinococcus cyst has ruptured into them from the neighboring tissues. In the sputum of pulmonary echinococcus, which is always bloody, and may, through communication with the liver, be bile or ochre colored, uninjured cysts with clear contents are occasionally present; in other cases the characteristic hooks may be seen, or shreds of membrane, which, when finely teased, allow the

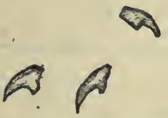

FIG. 6.—Echinococcus Hooks. (After *v. Hansemann.*)

parallel striations, typical of echinococcus membrane, to be recognized.

The hard ***yellow actinomyces granules*** (Figs. 7 and 8), which attract attention during the macroscopical examination, appear under the low power as round, unevenly knobby, finely granular objects, resembling a mulberry. When crushed under a cover glass, and examined with the high power, they have a very characteristic appearance. From a thick centre, composed of a mass of threads, radiate numerous glistening fibres, which branching many times, end in a bulbous enlargement (actinomyces clubs). In the central thready mass are often groups of spiral, rod-shaped, and coccus-like objects. The gray kernels resembling clumps of mucus, which are present alongside of the characteristic actinomyces clubs, are softer in consistency than these, and are composed merely of branched fibrils. (For stain-

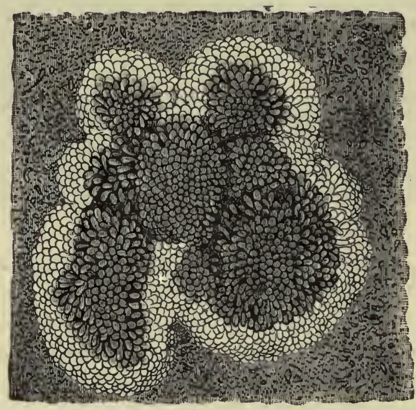

Fig. 7.—Actinomyces Granules (Low Power).
(After *v. Jaksch.*)

ing, *Gram's* method, with eosin as a counterstain, is suitable, the fibrils appearing bluish-black, and the bulbous ends red.)

In ***mycosis of the lungs*** small, grayish-black granules are found, which consist of mold fungi (aspergillus and mucor species).

The unstained specimen gives, in addition, information concerning the cellular elements of the sputum, and the presence of elastic fibres and crystalline bodies.

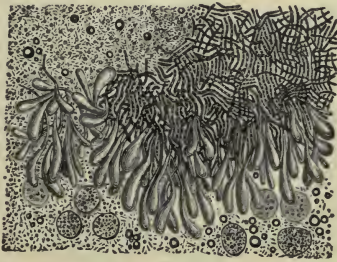

FIG. 8.—Actinomyces Granules (Unstained Specimen). (After *v. Jaksch.*)

Cellular Elements of the Sputum

1. ***Epithelial Cells.***—Inasmuch as the sputum represents the secretion of different portions of the respiratory tract, epithelial cells from every portion may be present in it. There may be:

(*a*) *Large polygonal squamous cells* coming from the mouth, pharynx, or vocal cords. These squamous cells are frequently covered with coal pigment.

(*b*) *Cylindrical cells*, which are occasionally ciliated. These may come from the pars respiratoria of the nose, from the larynx, or the bronchi.

(*c*) *Alveolar Epithelial Cells.*—These, when present, are always greatly degenerated, and cannot, as a rule, be recognized with certainty as such. By alveolar epithelial cells are meant round, oval, or polygonal, mono- or polynuclear cells, approximately five to six times the size of a leucocyte. Their protoplasm is frequently filled with highly refractive fat drops or faintly glistening myelin drops, which may coalesce to larger drops, and then produce the characteristic myelin forms. Further, black coal pigment is often seen in these cells (pigment cells). Under the heading of alveolar epithelium, cells containing reddish-brown pigment granules, the so-called heart-failure cells, are frequently included. These appear in great numbers in the sputum in brown induration of the lungs. This pigment, called hemosiderin, comes, as does hematoidin, from blood pigment; but, in contrast to hematoidin, contains iron.

Recognition of Hemosiderin.—Allow a fleck of sputum containing heart-failure cells to dry on a cover-glass, fix and drop on it a little 2 per cent. potassium ferrocyanide solution, to which 1 to 3 drops of HCl have been added. After half to one hour the pigment granules will be stained blue.

2. **Leucocytes.**—These are present in varying numbers in every sputum and in large quantity, as the principal ingredients of pus. They are usually more or less degenerated, being, as a rule, poly-morpho-nuclear, and showing generally neutrophilic granulation. Numerous eosinophilic leucocytes are found in the sputum of asthmatic patients. (Staining according to *May* and *Gruenwald*, cf. p. 258, gives a good picture.) The leucocytes contain frequently, like the so-called alveolar epithelium, pigment granules, coal pigment, as well as altered blood pigment. It is generally recognized now that the so-called heart-failure cells are not merely alveolar epithelial cells,

but that, on the contrary, the majority of them are leucocytes.

3. ***Red Blood Corpuscles.***—Isolated red blood cells are present in every sputum, and have no diagnostic significance. They point to hemorrhage in the respiratory organs only when they are present in great numbers. They may be perfectly intact both in form and color, or they may appear altered, being swollen, or crenated, or having lost their pigment (shadow corpuscles).

Elastic Fibres—(Fig. 9)

The material to be examined for the presence of elastic fibres should be taken from the opaque purulent portions of the sputum. To facilitate the search for elastic fibres the material to be examined should be mixed with a drop of 10 per cent. potassium hydrate solution, covered with a cover glass, and slightly warmed over a small flame. By this means the cellular elements will be decomposed, while the elastic fibres remain unchanged. When the search made in this manner is unsuccessful, several clumps

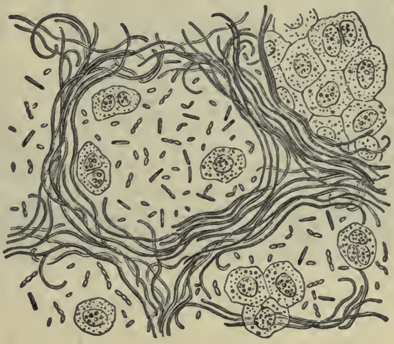

FIG. 9.—Elastic Fibres from the Sputum. (After *v. Jaksch.*)

of sputum should be put in a test-tube, an equal quantity of 10 per cent. potassium-hydrate solution added, and the mixture boiled until it appears homogeneous; then diluted with four times as much water and centrifuged. Smears should be made from the sediment and examined with a magnification of about 300.

The elastic fibres appear highly refractive, characteristically wavy, clear cut, double contoured, and frequently branching. They lie singly, in bundles of longitudinal fibres, or have a net or mesh like (alveolar) arrangement.

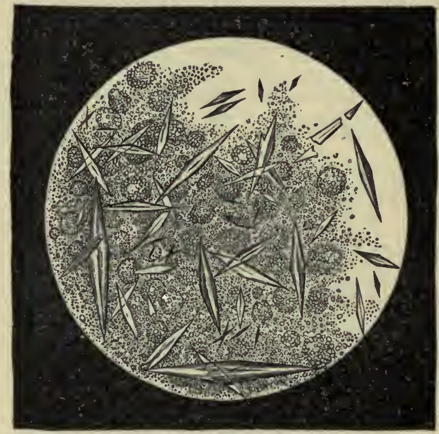

Fig. 10.—Leyden Crystals (Magnified 300 Times).

It must be remembered that elastic fibres coming from food may be present in the sputum. Only when elastic fibres in alveolar arrangement are found can it be said with certainty that they come from the lungs.

Crystalline Bodies

Of the crystalline bodies, *Charcot-Leyden* crystals have particular significance (Fig. 10). These appear as clear, glistening, pointed, octahedra, resembling spermin crystals in appearance. They are present in particularly large numbers when the sputum has stood for a time exposed to the air. Their formation may be observed microscopically in fresh cover-glass specimens.

In addition, fatty acid needles may be present, which can be distinguished from objects resembling them—for example, elastic fibres, by the fact that, when the slide is carefully warmed, they melt and coalesce, forming fat drops. Further, crystals of calcium oxalate, triple phosphate, and cholesterin, as well as of leucin and tyrosin, may be seen; and, finally, hematoidin crystals may be present in the form of reddish-yellow or ruby-red rhomboid plates, and wavy needles which lie free or radiate in tufts from the plates.

BACTERIOLOGICAL EXAMINATION OF THE SPUTUM

Preparation of the Sputum for Examination

Only such sputum is suitable for bacteriological examination as surely comes from the diseased air-passages. For examination the true bronchial or lung sputum must be separated from the secretions which have become mixed with it during its passage through the upper respiratory tract, as the latter frequently contain exactly the bacteria which are significant of pulmonary diseases.

The *Pfeiffer* and *Koch-Kitasato* methods serve this purpose. According to *Pfeiffer's* suggestion the sputum is poured into a sterile Petri dish, which is placed over a dark background, and a thick opaque portion is taken from it and placed upon the cover of the dish. This portion is thoroughly spread out by the aid of two platinum wires, and a distinctly purulent particle (the nucleus) is isolated.

According to the *Koch-Kitasato* method a sputum ball is washed in a series of dishes filled with sterile water, which is thoroughly stirred with a heavy platinum needle. In this manner the sputum ball quickly becomes smaller, and finally separates into minute particles, from which a small pus kernel is taken for examination. *Czaplewski* has modified this method by shaking the sputum ball in three successive tubes of peptone water.

The fleck, having in one way or another been freed from the adherent mucus and bacteria, which it has collected in its passage through the upper respiratory tract, may now be used for the preparation of smears, for planting cultures, or for animal inoculation.

I. ***Examination of Stained Smears.***—With the aid of a platinum wire the fleck is carefully smeared on cover glasses held in cornet forceps, either at once or when the sputum contains much fibrin, after the addition of a drop of sterile water. After the smears have been dried in the air, and have been fixed by passing three times through the flame, they are stained as follows:

1. According to one of the methods for staining tubercle bacilli (cf. p. 329).

2. With carbol fuchsin. Dilute carbol fuchsin is dropped on the smear, heated to the steaming-point over a small flame, and at once washed off, as the details are hard to recognize in too deeply stained specimens.

3. According to *Gram* (cf. p. 330). The first mentioned stain serves merely for the detection of tubercle bacilli. The smear, stained with dilute carbol fuchsin, gives information concerning—

(*a*) The origin of the sputum.

(*b*) The bacteria present other than tubercle bacilli.

(*c*) The value of the bacteriological findings.

It is not always possible to determine the origin of the sputum with certainty from the microscopical picture, although the epithelial cells present in the sputum, which have been called by *Czaplewski* "guide cells," are of assistance.

When the secretion comes from the mouth, pharynx, or nasopharynx, numerous large squamous cells, which are usually thick with bacteria, are seen in the smear, and in addition more or less numerous pus cells, depending upon the stage of the inflammation.

Nasal secretion which has been aspirated and coughed out shows, besides a varying quantity of leucocytes, cylindrical cells which are occasionally ciliated, as well as squamous cells from the pharyngeal sputum, which is usually mixed with it. Further, the rich bacterial flora of the nose and pharynx is always represented by a great number of different micro-organisms.

Bronchial and pulmonary sputum contains, besides pus cells, cylindrical epithelial cells and the pigment cells, which are especially characteristic of it.

Inasmuch as nearly all the micro-organisms which come into question in sputum examination, with the exception of tubercle bacilli, are easily stained with dilute aniline dyes, they can be clearly seen in the specimens stained with dilute carbol fuchsin.

When a certain kind of bacterium is found by repeated examination in large numbers in sputum coming from the deeper air-passages, it is proper to assume that these bacteria have an etiological connection with the disease. When, on the contrary, the specimen shows a mixture of different kinds of bacteria, the findings have no diagnostic value, unless it is determined that these were present before the expectoration of the sputum, and are not due to subsequent contamination.

The specimen stained by *Gram's* method aids in find-ing *Gram*-positive bacteria, which, because of their dark staining, are prominent against the brown background of decolorized cellular elements.

Further, it shows how the bacteria seen in the carbolfuchsin specimen act toward the *Gram* method, and thus aids in identifying them.

II. For ***cultural examination*** plain agar, glycerine agar, blood agar, blood serum, and bouillon are, as a rule, used; other culture media, as gelatine, potato, etc., are but exceptionally used. The washed fleck is smeared either immediately or after being floated in physiological salt solution. When the presence of many germs capable of development is suspected, the same fleck is smeared on a number of culture media to obtain isolated colonies.

III. ***Animal Inoculation.***—White mice, guinea-pigs, and rabbits are the test animals most frequently used in sputum examination. The washed sputum fleck is either introduced directly into a pocket under the skin, or, after being floated in a sterile 0.85 per cent. sodium chloride solution, is injected subcutaneously or intraperitoneally.

Detection of Tubercle Bacilli. (Plate II, Fig. C.)—Material for smears should always be taken from a number of suspected places in the sputum, the so-called kernels being especially sought after. In specimens prepared after the method described on p. 330, the tubercle bacilli are stained red, while the other bacteria and cellular elements are blue. The tubercle bacilli present themselves as slim rods of varying length, and do not always appear perfectly straight, but are often slightly curved. They lie in groups, singly, or in pairs, which may be parallel or at right angles to one another. They are often of uneven thickness, or irregularly granular. Colorless spots are seen between the stained granules, so that the bacilli resemble a string of pearls. Further single, small, blue to blackish red "venous" stained objects are found, which are thought to be fragments of bacilli, and which *Spengler* has called "splinters." Fibril forms, with true branching and bulbous ends, have been very rarely observed in sputum.

The number of bacilli found in a smear gives no clue to the course of the disease, for their number is most variable, both in different portions of the same sputum, and in sputum expectorated at different times of the day. Nevertheless, the question of counting tubercle bacilli may arise, in which case the method of *Gaffky* or that of *Czaplewski* may be used.

GAFFKY'S SCALE

1 = 1—4 bacilli in an entire specimen.
2 = on the average only 1 bacillus in a number of fields.
3 = " " 1 bacillus to a field.
4 = " " 2—3 bacilli to a field.
5 = " " 4—6 " " "
6 = " " 7—12 " " "
7 – " " quite a few bacilli to a field.
8 = " " numerous " " "
9 = " " very numerous bacilli to a field.
10 = a very large number of bacilli in every field.

CZAPLEWSKI'S METHOD

"In the numerator of a fraction is written the number of bacilli which are counted in a field; in the denominator, the number of fields counted. If in one or more entire smears only a few bacilli are counted, then the denominator is written as a Roman numeral, which represents the number of smears examined. For example:

$\frac{6}{1}$ = 6 bacilli in a field.

$\frac{\infty}{1}$ = innumerable bacilli in a field.

$\frac{1}{5}$ = 1 bacillus in five fields.

$\frac{2}{1}$ = 2 bacilli in an entire smear.

$\frac{1}{\mathrm{VI}}$ = 1 bacillus in six entire smears,

and so forth. It is perhaps well to express the minimum and maximum number observed $\left(\text{for example } \frac{0-6}{1}, \text{etc.}\right)$ and also the average found in a number of fields

$$\left(\text{for example } \frac{0-6}{1} = \frac{3}{1}\right)$$

"It should, however, be mentioned that the diameter of a field varies with change of objective, ocular, or tube length, and, therefore, the results are of value only when arrived at with the same optical combination."

Sedimentation.—This procedure, first used by *Biedert*, has for its object, by liquefying the sputum and rendering it homogeneous, the collection in the sediment of the isolated and scattered bacilli, that they may be more easily detected. A great number of sedimentation methods have been proposed.

Beitzke, who has tested the various methods, recommends especially the *Muehlhaeuser-Czaplewski* method, which is carried out in the following manner:[1]

"The sputum is placed in a cylindrical glass, about four times as much 0.2 per cent. sodium hydrate solution added, the glass closed with a rubber cork and shaken vigorously for a minute. This is often sufficient to produce an even, thinly liquid, and no longer mucoid, substance, in which no large flecks are visible. When this is not sufficient, more alkali is added, the glass again vigorously shaken, and so on, until such a fluid is produced. As a rule, not more than eight times as much alkali as sputum will be needed, though I have occasionally found it necessary to dilute twelve times. When the sputum has in this way wholly lost its mucoid aspect, and has become entirely liquid, it is poured into a porcelain or enamel dish, and under constant stirring heated to the boiling-point.

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[1] "Hygienische Rundschau," 12, No. 1.

"When the sputum has become entirely homogeneous, one or two drops of phenol-phthalein solution are added, then, drop by drop, under vigorous stirring, 5 per cent. acetic acid solution, until the red color disappears. If the mixture is not stirred energetically enough, it is easy to add too much acetic acid, in which case the whole purpose of the procedure will be thwarted by the heavy precipitation of mucin. This is sure to be the case when the liquid had even the slightest mucoid character before its neutralization. After its neutralization the liquefied sputum is diluted with water, or with two parts alcohol, and either allowed to stand or is centrifugalized." It is possible to centrifugalize the entire quantity in a single centrifuge tube by continually pouring off the fluid from the sediment and filling the tube anew, until the entire sputum has been used. From the sediment obtained in this manner smears are made and stained.

The ***cultivation of tubercle bacilli*** from the sputum hardly enters into consideration for diagnostic purposes, as it is too tedious and frequently fails. *Kitasato* first succeeded according to the following method: The patient is requested to thoroughly cleanse his mouth with a gargle, and then to expectorate into a sterile dish. A sputum ball is washed in the manner described above, torn apart under water in the last dish, a particle taken from its centre and examined in a stained smear. If the sputum contains tubercle bacilli in pure culture, glycerine agar and blood serum tubes are inoculated with a second particle. The cotton plugs are cut short and pressed into the tubes, and in order to prevent drying of the culture media the tube is sealed with a rubber cap, still moist from the sublimate solution in which it has been disinfected. After two weeks' growth the colonies are visible as moist, glistening, smooth, round flecks.

W. Hesse[1] recommends the following procedure for the cultivation of tubercle bacilli from sputum. The sputum raised from the depth of the lungs, when possible without the addition of saliva, is expectorated into a sterile Petri dish. A portion of the sputum the size of a pea is placed on the culture media (plate culture) and divided into twenty to thirty small flecks.

Preparation of the Culture Media.—One part agar-agar, 3 parts glycerine, 96 parts water, are mixed, filtered, and placed in test-tubes of a capacity of 50 cc, which must be of resistant glass containing no alkali, so that each tube contains 20 cc. The tubes containing the culture media are steam sterilized for about three hours. The culture media must have the same alkalinity as the sputum which is to be examined. For this purpose six tubes are taken; to five of them is added decinormal KOH in quantities of 0.2, 0.5, 1.0, 2.0, and 5.0, from which six plates are made and inoculated in the above manner. It is then to be expected that one of the plates will have the desired alkalinity. Or the following method is employed: To five of six test-tubes containing 20 cc of water, decinormal KOH is added in quantities of 0.2, 0.5, 1.0, 2.0, and 5.0, and a drop from each is placed on a strip of litmus paper. One of the 6 drops will color the paper, approximately as blue as does the sputum, and will show how much alkali should be added to the media to give it practically the same reaction as the sputum.

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[1] *Centralblatt f. Bakt.*, xxxv. No. 3.

A strip of asbestos is placed between the cover and bottom of the inoculated dishes; they are held tightly closed by a rubber band, and placed with the culture media uppermost in an incubator. After one to two days the plates are tested by means of *Klatsch*-preparata made in the following manner: a sterile cover glass resting over the opening of a test-tube is carefully pressed upward against the surface of the culture where there is a sputum fleck. The cover glass is then lifted from the culture media with a platinum wire and grasped with forceps. The growth of the tubercle bacilli can be detected in the *Klatsch*-preparata. The colonies may be distinctly seen after a few days' growth with the low power, and after a few weeks with the naked eye.

More reliable results are to be obtained from animal inoculation than from cultural methods. Half-grown guinea-pigs, weighing about 250 grammes, are used, as they are more sensitive than older animals. They are inoculated subcutaneously, either on the abdomen or on the thigh, by introducing a washed fleck into a niche under the skin, or by injecting the fleck suspended in physiological salt solution. The inoculation must be made in an aseptic manner, after the skin has been shaved and washed with alcohol. The subcutaneous inoculation has the advantage over the intraperitoneal that it enables one to follow, step by step, the advance of the tuberculosis.

The tubercular process produces at first an infiltration at the site of inoculation, next attacks the regional lymph-nodes, and then spreads to the viscera.

After three to four weeks tubercle bacilli may be detected in the pus of an extirpated node, or in the fistula at the site of injection. If the animal is killed four to six weeks after inoculation, the autopsy reveals the following conditions: infiltration at the site of injection, the neighboring lymph-nodes enlarged and caseated, the internal lymph-nodes enlarged, and more or less numerous tubercles in the viscera, especially in the spleen and liver. The search for tubercle bacilli must never be omitted. For this purpose a tubercle is placed with a scalpel or platinum wire on a slide, crushed and smeared over it, and stained in the usual manner. As a rule only a few isolated bacilli are found in the tubercles. It is easier to obtain cultures of tubercle bacilli from tubercles than from sputum. For this purpose a series of blood-serum tubes are inoculated and closed with sterile rubber caps. The material to be inoculated must be crushed between two sterile slides or scalpels, and thoroughly mixed with the culture media. After two weeks at the earliest, dry, yellowish-white flakes appear, from which finally a pure culture develops, forming a closely coherent, wrinkled membrane.

Differential Diagnosis.—Although acid-fast bacilli other than tubercle bacilli may appear in sputum, their presence is so very rare that this does not detract from the diagnostic value of stained smears. They have, however, been observed in cases of pulmonary gangrene, bronchiectasis, and putrid bronchitis. Therefore, particular care should be taken in making a diagnosis when such conditions are present. These acid-fast bacilli may, to be sure, differ in their form from tubercle bacilli. They are usually slimmer and straighter than tubercle bacilli and slightly pointed at the ends, but these differences, when compared with the varying appearance of tubercle bacilli, are too slight to allow a certain diagnosis to be made. Although the other acid-fast bacilli are frequently more easily decolorized by absolute alcohol than tubercle bacilli, this is by no means a constant characteristic, and should not be relied upon in making a differential diagnosis. In cultures they differ from tubercle bacilli in that they develop more quickly, and at room temperature on artificial culture media. After twenty-four to forty-eight hours' growth on glycerine agar, white, glistening colonies the size of a pin's head have appeared, which gradually coalesce, forming a white, creamy coating. After longer growth, the lustre disappears and the surface looks dry. At room temperature an orange-yellow pigment gradually forms. Animal inoculation presents the surest means of differentiation, as the other acid-fast bacilli never produce the typical appearance of tuberculosis. To be sure, when injected with butter into the peritoneal cavity of guinea-pigs, they excite, in addition to a fibrinous peritonitis, changes which resemble, macroscopically, tubercular nodules (true tubercles) but which, when examined histologically, differ widely from them, in that they show a more exudative than productive character, and, further, that *Langhans*' giant cells, as well as epithelioid cell nests, are lacking. Finally, in contrast to the true tubercular nodules they contain usually numerous acid-fast bacilli.

Further, lepra bacilli, which are also acid fast, must be considered in making a diagnosis. They differ, however, from tubercle bacilli, in that they stain easily with watery gentian violet and fuchsin solutions (cf. p. 332). They are rarely seen singly, but lie usually within the cells arranged in a group resembling a bunch of cigars.

In this case, also, the final decision depends upon the results of cultural tests and animal inoculation. The negative result of both is evidence in favor of leprosy, as, up to the present time, attempts to cultivate lepra bacilli, or to infect animals with them, have failed.

The other bacteria which appear in the sputum, both as independent exciters of disease and as producers of mixed infection in tuberculosis, are seen in the specimens stained with dilute carbol fuchsin, and according to *Gram.*

Pneumococci (Plate II, Fig. D, and Plate III, Fig. E)

Microscopical Examination.—Pneumococci present themselves, as a rule, as diplococci, which are pointed on one side, usually the outer, less frequently the inner, while the opposite side appears rounded (lancet form). They have a distinct capsule, and stain according to *Gram.* They frequently form short chains. The picture which the stained smears present is often so characteristic, that from the microscopical specimen alone the diagnosis "pneumococci" can be made. In other cases, however, cultures and animal inoculation must be used for their identification.

Cultivation.—On glycerine agar pneumococci develop small colonies resembling dew-drops; in bouillon they grow frequently in long chains. The capsules fail to appear, as a rule, in the growths on artificial culture media. They are, however, not infrequently found in specimens made from blood-serum cultures.

Animal Inoculation.—The most suitable test animals are rabbits and white mice. The sputum fleck is dissolved in physiological salt solution and injected subcutaneously, about 0.1 cc for a mouse, and about 0.5 to 1 cc for a rabbit. After twenty-four to forty-eight hours the animals die of pneumococcus septicemia. In the blood from the heart and in the viscera, innumerable pneumococci with capsules are found. The easiest method of isolating pneumococci from the sputum is animal inoculation.

Streptococci

Microscopical Examination.—Streptococci are arranged in chains of varying length, whose individual components are spherical. Diplo forms, which are found to be streptococci only after cultivation, are frequently present in the sputum. They stain according to *Gram.*

Cultivation.—On agar they grow rather slowly in very small, delicate, transparent colonies. With the low power, the centre appears finely granular and darker than the periphery; the latter is either regular and smooth, or may be irregular and frayed, allowing the individual chains of streptococci to be seen. In smears taken from agar cultures, the chain formation is frequently absent. In bouillon they produce, as a rule, a flaky precipitate without rendering it cloudy, and develop long chains (*Streptococcus longus*); in rarer cases they render the bouillon cloudy, and form shorter chains (*Streptococcus brevis*). They do not liquefy gelatine.

The *Streptococcus mucosus* is surrounded by a distinct capsule and is characterized by its colonies.

For the differential diagnosis between streptococci and pneumococci ox-gall is used; 0.5 cc of ox-gall are added to 2 cc of the bouillon culture. If pneumococci or streptococci are present, the cloudy mixture will clear up after a few minutes, while the other streptococcus species will not change the appearance of the gall, because of the bacteriolytic action of the gall toward the pneumococci and the streptococcus mucosus.

Animal inoculation is not necessary in making a diagnosis.

Staphylococci

The *Staphylococcus aureus*, or *albus*, is usually present in the sputum, more rarely the *Staphylococcus citreus.*

Microscopical Examination.—Staphylococci appear as round cocci, usually arranged like a bunch of grapes, and stain according to *Gram.*

Cultivation.—On agar they produce large, round, slightly elevated, non-transparent colonies of yellow (*Staphylococcus aureus*), white (*Staphylococcus albus*), or lemon-yellow (*Staphylococcus citreus*) color. All true staphylococci liquefy gelatine and render bouillon cloudy. Animal inoculation is unnecessary in making a diagnosis.

Micrococcus Tetragenus

The *Micrococcus tetragenus* appears in the sputum only as producer of mixed infection in tuberculosis.

Microscopical Examination.—The cocci are round or oval, of varying size, and lie in tetrads within a capsule. They stain according to *Gram.*

Cultivation.—On agar they produce white, non-transparent, moist, glistening colonies, at the periphery of which, when examined with the low power, the tetrad arrangement can be seen. On gelatine plates they appear, at first, as small white points, which soon increase in size and cover the gelatine with a glistening, porcelain-like coating. Bouillon remains clear, though a moderate precipitate forms.

Animal Inoculation.—White mice are particularly sensitive, and die of septicemia within a few days after the infection.

Micrococcus Catarrhalis (Plate III, Fig. F)

The *Micrococcus catarrhalis* appears in the sputum as the exciting cause of bronchitis and broncho-pneumonia, particularly in children, but also in adults, either alone or together with other exciters of inflammation, especially streptococci and influenza bacilli.

Microscopical Examination.—It appears as a diplococcus, or a tetracoccus, but never forms chains. It resembles the gonococcus very closely, both in form and position; it is, however, much larger. In the acute stage the cocci lie frequently outside the cells, but later often within the leucocytes, closely grouped around the nucleus. Like the gonococcus, it is decolorized by *Gram.*

Cultivation.—It grows on neutral or slightly alkaline agar, but more luxuriantly on blood agar and serum agar. After twenty-four hours' growth it produces slightly elevated, grayish-white, glistening colonies, having the crumbling, gritty consistency of mortar. Examined with the low power, they are yellowish-brown in color, unevenly granular, and have a very irregular, ragged outline. Gelatine is not liquefied. It produces a precipitate in bouillon without clouding it, and after some days a scum appears on its surface.

Influenza Bacillus (Plate IV, Fig. G)

Microscopical Examination.—Influenza bacilli are very small, ovoid rods, which are decolorized by *Gram.* They lie frequently within the cells, and appear usually in great quantities in the sputum, so that the smear looks as if it had been powdered with them. In stained sputum smears they resemble closely the *Bacillus pyocyaneus*, from which, however, they are easily distinguished by cultural methods (cf. *B. pyocyaneus*)

Cultivation.—Influenza bacilli do not develop on plain agar. They grow best on blood agar and in blood bouillon. On the former they produce clear colonies, resembling dew-drops, which show no tendency to coalesce. When closely crowded, they run together, forming larger drops, though even then the individual colonies may be distinguished. In blood bouillon they produce delicate white flakes.

In cultural examination of the sputum, besides blood agar, plain agar is, as a control, inoculated with bouillon in which the material for examination is suspended. When influenza bacilli are present, there must be no growth on the plain agar, while on the blood agar the above described colonies appear.

Animal Inoculation.—Influenza bacilli do not infect the usual test animals.

Streptobacilli have been found as producers of mixed infection in tuberculosis. They belong to the group of influenza bacilli, and have the same cultural characteristics, but differ morphologically, in that they are considerably larger, and have a surrounding capsule.

Diplobacillus of Friedlaender (Pneumobacillus)

Microscopical Examination.—The pneumobacilli are plump rods with rounded ends, varying greatly in size and form, often resembling cocci. They lie in pairs, and possess usually a distinct capsule, which is especially conspicuous in sputum smears, in contrast to those made from cultures. They are decolorized by *Gram.*

Cultivation.—They grow at room or incubator temperature upon the usual culture media, and produce either grayish-white, moist, glistening and slimy, or firmer, nontransparent colonies. They do not liquefy gelatine, but frequently, after longer growth, stain it brown. They ferment grape sugar, but do not coagulate milk.

Animal Inoculation.—White mice die within twenty-four to forty-eight hours after subcutaneous or intraperitoneal inoculation. Numerous diplobacilli having capsules are found in the blood and viscera.

Bacillus Pyocyaneus

The *Bacillus pyocyaneus* has been reported as a producer of mixed infection in tuberculosis. The sputum is stained by its pigment blue or bluish-green, and has a characteristic aromatic odor.

Microscopical Examination.—The bacilli appear as small, slim rods, which are decolorized by *Gram.*

Cultivation.—On culture media *B. pyocyaneus* produces a pigment which stains the entire media. On agar its colonies are round, with a smooth circumference; on gelatine they are flat, with an irregular border, and are soon surrounded by a liquefied area. Bouillon is markedly clouded, milk coagulated and peptonized. The *B. pyocyaneus* differs from the influenza bacillus in that it is easily cultivated on the usual culture media, produces pigment, and is motile.

Animal Inoculation is not necessary for diagnostic purposes.

Bacillus of Bubonic Plague

Plague bacilli (*B. pestis*) are found in the sputum of primary pulmonary plague, and in the pneumonia and terminal pulmonary œdema of severe plague septicemia. Patients convalescing from pulmonary plague may expectorate virulent pest bacilli.

Microscopical Examination.—Plague bacilli are found in the sputum in pure culture, or frequently together with other bacteria—namely, diplococci and streptococci. The smears are best fixed, according to *Sobernheim*, in absolute alcohol, which is dropped on the cover glass, allowed to act about a minute, then lighted, and quickly extinguished. They are stained with dilute borax methylene blue. The plague bacilli appear as short, oval rods, which are stained more intensely at the ends than in the middle (polar staining). Their form, however, varies greatly. In addition to the typical rods, short, oval ones (coccus type), as well as long ones (rod type), and often involution forms in the shape of irregularly bordered ovoids or discs, which stain poorly, and resemble yeast cells, are seen. The plague bacillus is decolorized by *Gram*.

Cultivation.—The reaction of the culture media must be neutral or slightly alkaline. Cultures on agar must be kept at 30° C., those on gelatine 20° to 22° C. The latter is particularly suited to the examination of sputum and other secretions which contain other bacteria in addition to the plague bacilli, for the plague bacillus develops well at 22° C., while the growth of the other bacteria is inhibited. Gelatine plates are inoculated in the same manner as are agar plates, by spreading the material to be examined in a thin film over the hardened gelatine. On agar plates, after twenty-four hours' growth, small colonies resembling dew-drops are visible, which, after forty-eight hours, appear transparent, with a prominent, darkly colored, granular centre, and a broad, delicate, irregular periphery. On dry culture media containing 3 to 4 per cent. of sodium chloride plague bacilli develop the characteristic involution forms. On gelatine, which they do not liquefy, they produce, after two to three days, yellow colonies, whose coarsely granular centre rises above the surface of the gelatine, and is surrounded by a delicate, clear, jagged border.

The stalactite formation in undisturbed bouillon cultures is characteristic.

Animal Inoculation.—The most suitable test animals are rats and guinea-pigs. The former are inoculated either subcutaneously, or on the uninjured conjunctiva, or by means of their food; the latter cutaneously by inunction on the shaved abdomen. This latter method gives especially good results in sputum examination. After one or two days the regional lymph-nodes become markedly swollen, and after four or five days death ensues. Material for cultures can be obtained from the buboes as early as twenty-four to forty-eight hours after the inoculation. The cultivated bacteria are identified by means of agglutination tests.

Typhoid bacilli are occasionally found in the sputum in bronchitis and pneumonia accompanying typhoid fever. In the cases in which they are detected, they are found either alone or together with streptococci, diplococci, and influenza bacilli. Anthrax bacilli appear in the sputum of pulmonary anthrax (wool-sorter's disease). *Bacterium coli* accompanying pneumococci has been very frequently detected in cases of pneumonia in nursing children. These bacteria are identified according to methods described elsewhere.

Sputum which is expectorated in a decomposed condition, as in pulmonary gangrene, bronchiectasis, and putrid bronchitis, has a rich bacterial flora. Besides the true exciters of inflammation, *B. fusiformis*, *proteus*, *pyocyaneus*, pseudo-diphtheria bacilli, occasionally acid-fast bacilli, etc., may be present.

Sputum which is expectorated when an empyema has ruptured into the lungs contains, usually, in addition to other micro-organisms, anaerobic bacteria.

CHAPTER V

EXAMINATION OF THE GASTRIC CONTENTS

General Characteristics

(*a*) ***Quantity.***—The filtrate of the gastric contents, obtained exactly one hour after the *Ewald* test breakfast (35 to 70 grammes of white bread and one cup of tea), gives an idea of the amount of the gastric contents, sufficiently accurate for practical purposes. This is normally, according to *Boas*, 20 to 25 cc.

A more accurate estimation of the total gastric contents is carried out according to *Strauss* in the following manner: First, a portion of the gastric contents is withdrawn, its quantity and specific gravity determined; a definite quantity of water is then introduced into the stomach, allowed to mix with the gastric contents, as much as possible withdrawn, and the specific gravity of the diluted gastric contents determined. The following formula gives the desired quantity:

$$X = \frac{V.S. + (a - V)\,S' - a}{S - S'},$$

in which S represents the specific gravity of the undiluted, S′ the specific gravity of the diluted contents, V the quantity of the diluted contents, and a, the quantity of water added.

(*b*) ***The odor*** of the gastric contents is normally slight. Even under pathological conditions there may be no marked odor, if the stomach was empty before its reception of the test breakfast. In cases of marked gastrectasis, there is often a strong pungent odor, due to volatile fatty acids (butyric acid, valerianic acid). Decomposition of an extensive carcinoma of the stomach produces a foul odor; in cases of ileus the odor is fecal.

(*c*) ***Color.***—Pure gastric juice, as well as the gastric contents after the test breakfast, is normally colorless. Frequently, however, a slight mixture of bile causes a yellow or greenish color. The presence of a larger amount of bile pigment produces a grass-green color (the bilirubin being converted by a longer stay in the stomach into biliverdin).

The change of color in pathological cases is most frequently due to the admixture of blood. Small streaks of blood on the surface of the gastric contents have no particular meaning, as they are usually caused by retching. A bloody coloration of the entire contents points to the presence of severe disease, and contraindicates further sounding.

(*d*) ***Consistency.***—The gastric contents after the test-breakfast have usually a thin, pappy consistency. When mixed with large quantities of mucus, they have a slimy consistency.

Inspection of the gastric contents gives information as to the extent of the action of the gastric juice. A distinction is made between absolutely undigested, partially digested, and well-digested gastric contents. When digestion is entirely absent, the gastric contents resemble the original meal lying in water. In partially digested gastric contents, more or less undigested food particles are visible. Occasionally, the formation in the receptacle of three strata may be noticed. The top stratum consists usually of mucus or coarse food particles (mostly undigested); the middle, the largest stratum, of fluid; the bottom of chyme. In the macroscopical examination of the gastric contents we must pay attention whether pus, blood, or stagnated food is present.

Qualitative Chemical Examination

1. **Reaction.**—The reaction of the gastric contents is determined in the usual manner with litmus-paper. It may be acid, neutral, amphoteric, or alkaline.

In the majority of normal and pathological cases the reaction of the gastric contents is acid.

The normal *acid* reaction of the gastric contents after the test-meal is due to:

1. Free hydrochloric acid.
2. Combined hydrochloric acid.
3. Acid phosphates.
4. Traces of organic acids (carbonic acid, lactic acid, acetic acid, butyric acid, etc.).

2. **Free Hydrochloric Acid.**—The term "free hydrochloric acid" is generally used in contradistinction to the term "combined hydrochloric acid." The hydrochloric acid has a marked affinity toward albuminoid substances and their digestive products with which it forms *acid*, loose combinations. In the first stage of the digestion the largest portion of the secreted hydrochloric acid is combined by albuminoid substances. Thus we term free hydrochloric acid the surplus left after the combination of the albuminoid affinities present.

The other tests for free acids are used also as tests for free hydrochloric acid, and are, therefore, described under that heading.

The tests for free hydrochloric acid may be divided into two groups:

(1) Tests characteristic of hydrochloric acid alone.

(2) Tests detecting all free acids, but which, in the

examination of the gastric contents, may be used as tests for free hydrochloric acid.

To the first group belongs *Günsburg's* test with phloroglucin-vanillin.

The reagent consists of:

Phloroglucin	2.0
Vanillin	1.0
Absolute alcohol	30.0

Three drops of the reagent are thoroughly mixed in a porcelain dish with an equal quantity of filtered gastric contents, carefully heated over a small flame (without reaching the boiling-point) until the mixture has entirely evaporated. A beautiful carmine mirror forms, especially at the edge. This mirror appears even with a dilution of 0.01 per cent. hydrochloric acid. With a dilution of 0.005 per cent. merely fine red streaks are produced. This reaction is not produced even by the most highly concentrated organic acids. As it is also very delicate, it is recognized universally as the surest and most reliable reaction for the detection of free hydrochloric acid.

According to *Boas* this reaction may be carried out with strips of filter-paper impregnated with the reagent. When such a reagent-paper is touched with 2 to 3 drops of gastric contents and carefully warmed over a flame, a carmine spot appears, which remains unchanged on the addition of ether.

It must be mentioned that *Günsburg's* reagent decomposes if kept a long time, and it is, therefore, advisable to test the solution with very dilute hydrochloric acid carrying out the reaction.

Of the reactions of the second group are to be recommended:

(*a*) THE TEST WITH KONGO-PAPER—The red color of the reagent-paper is changed into blue by free hydrochloric acid. The more free acid is present, the stronger becomes the shade of blue. With but faint traces of free hydrochloric acid the color changes to light black-blue.

(*b*) THE TEST WITH METHYL VIOLET.—Weak hydrochloric acid (under 0.5 per cent.) colors a violet solution of this dye blue. Organic acids change the color of the solution only when more concentrated (over 0.5 per cent.).

Performance of the Test.—To 5 to 10 cc of water in a test-tube 2 to 3 drops of a concentrated watery solution of methyl-violet are added, which must give the water a distinct violet color. To another test-tube, containing 5 to 10 cc of gastric contents, the same amount of dye is added as was added to the water, and the two solutions are compared. If the gastric contents appear blue, free hydrochloric acid is present.

(*c*) THE TEST WITH DIMETHYLAMIDOAZOBENZOL.—A 0.5 per cent. alcoholic solution of this dye is used as reagent. Hydrochloric acid colors the orange-yellow solution bright red.

Performance of the Test.—To 3 to 5 cc of the filtered gastric contents 3 drops of the solution are added. If even a faint trace of free hydrochloric acid (0.002 per cent.) is present, the solution becomes fiery red. Organic acids produce this reaction only when more concentrated than 0.5 per cent., and then only in the presence of albumin, peptone, or mucus. Neither does loosely combined hydrochloric acid produce this reaction.

These color tests are more or less sensitive, but they do not give absolutely reliable results, and when hydrochloric acid is present only in small amount, it is hard to distinguish it by them from organic acids.

3. **Lactic Acid.**—Of the two kinds of lactic acid, fermentation lactic acid (optically inactive), and meat lactic acid (optically active), the first only need be considered in examining the gastric contents. It is formed as a product of the fermentation of carbohydrates caused by bacteria (*Bacterium acidi lactici*).

It is detected by the following reactions:

The Simple Ferric Chloride Test.—To 20 to 30 cc of water 3 to 5 drops of liquor ferri sesquichlorati is added. The water assumes a hardly noticeable yellow tinge. The thus obtained reagent is divided into two test-tubes and into one of the test-tubes the gastric contents, which are to be examined, are added, drop by drop. If lactic acid is present, the solution changes its color to canary-yellow. The change of the color becomes more visible by holding the two test-tubes against a white background.

(*b*) *Modification According to H. Strauss.*—Five cc of gastric contents are shaken with 20 cc of alcohol-free ether. After the solution has settled, 1 part (5 cc) of the ether is poured off, diluted with 4 parts of water and 2 drops of a ferric chloride solution (1 : 9), and vigorously shaken. When about 0.1 per cent. of lactic acid is present a distinct green color appears, when less is present a fainter green color appears.

4. **Volatile Fatty Acids.**—Of the volatile fatty acids, particularly acetic and butyric acids are to be considered in the examination of the gastric contents. They are either introduced with the food or are formed as products of abnormal carbohydrate fermentation. It is only in the latter case that they are of diagnostic significance.

As a preliminary test for the presence of volatile fatty acids the following simple test (adequate for practical purposes) may be used. About 10 cc of the gastric contents are heated in a test-tube, at the upper end of which there is a small strip of moist blue litmus-paper. When volatile acids are present the litmus-paper turns red (*Leo*).

The following method is more definite: To 15 to 20 cc of gastric contents 1 gramme of sodium sulphate is added, and the mixture thoroughly shaken two or three times, each time with 50 cc of ether. The ether is poured off and distilled. A fluid residue remains, which, if organic acids are present, has a distinct acid reaction and a characteristic odor. The residue is then divided into two equal portions, with which the tests for acetic and butyric acids are carried out.

(*a*) *Detection of Acetic Acid.*—The liquid is taken up with water, neutralized exactly with a dilute soda solution, and a drop of ferric chloric added. If acetic acid is present the liquid turns blood-red, and, when boiled, throws down a brownish-red precipitate of basic ferric acetate.

To be sure, formic acid produces the same reaction, but this fact does not alter the diagnostic value of a positive reaction, since formic acid can be present in the gastric contents only as a product of acid fermentation.

(*b*) *Detection of Butyric Acid.*—The second portion of the ether residue is dissolved in 2 to 3 drops of water, and treated with a very small particle of calcium chloride. The butyric acid separates (because of its insolubility in salt solutions) into little drops which float on the surface, and which have the characteristic odor of butyric acid.

5. ***Pepsin and Pepsinogen.***—Pepsin, the proteolytic ferment of the gastric juice, is formed from pepsinogen, the specific product of the peptic cells of the gastric glands, by the action of acids. The conversion of pepsinogen into active pepsin is produced with special rapidity by the action of hydrochloric acid.

Upon this fact depends the detection of pepsin and pepsinogen. If the gastric contents contain free acid, and at the same time digest albumin, pepsin is present. When the gastric juice contains no free acid, only pepsinogen can be present. Such gastric contents must have the power to digest albumin after the addition of a sufficient quantity of hydrochloric acid. If this is not the case, pepsinogen is also absent.

Performance of the Digestive Test.

1. *According to Mett.* Egg-albumin is filtered through a piece of gauze into a small beaker or wide test-tube, and short glass tubes having a lumen of about two millimetres are slowly dropped into it. Air-bubbles, which rise in the tubes, are allowed to escape, aided by gentle tapping. The vessel containing the tubes is then placed in a boiling-water bath for five to ten minutes. The flame is then removed, and the glass allowed to cool slowly for several hours. The test-tube is now broken, and the small tubes which are filled with, and imbedded in, the coagulated egg-albumin, are cut out and preserved either in glycerine or chloroform water.

One tube is used for each test. It is first washed with water, then put into a test-tube containing 10 cc of filtered gastric contents, and placed in an incubator for twenty-four hours. If during the chemical examination hydrochloric acid was found to be absent, 1 or 2 drops of official hydrochloric acid are added; if pepsin is present, a portion of the albumin will be digested at the end of twenty-four hours. This test serves at the same time for the quantitative estimation of pepsin, the length of the digestive column of egg-albumin (in millimetres) being proportional to the amount of pepsin.

2. *Jacobi's* method. The principle of the test consists in the fact that a ricin solution, which is made cloudy by

the addition of HCl, is clarified by the addition of pepsin. First several solutions are prepared of filtered gastric juice, which have been previously diluted with water and the lowest degree of dilution is determined at which a certain quantity of ricin solution will clarify.

Reagents needed:

1. A 1 per cent. ricin solution in a 5 per cent. NaCl solution (0.5 ricin in 50 cc salt solution prepared extemporaneously).
2. A decinormal HCl solution.

Procedure.—Eight test-tubes of equal calibre are filled each with 2 cc of the slightly cloudy and filtered ricin solution and with 0.5 cc of a decinormal HCl solution. The addition of the HCl produces a milky cloudiness. The test-tubes, numbered 1 to 8, are put in a test-tube-holder. Tubes 1 and 8 are for controlling purposes, 2 to 7 contain the gastric juice in the various degrees of dilution as follows:

Tube 2. 1.0 cc of the undiluted gastric juice (dilution 1:1)
Tube 3. 0.2 cc of the undiluted gastric juice (dilution (1:1)
Tube 4. 1.0 cc 10 times diluted gastric juice (dilution 1:10)
Tube 5. 0.2 cc 10 times diluted gastric juice (dilution 1:50)
Tube 6. 1.0 cc 100 times diluted gastric juice (dilution 1:100)
Tube 7. 0.5 cc 100 times diluted gastric juice (dilution 1:200)

In order to obtain equal volumes, to each test-tube distilled water is added whatever is missing to 3.0 cc: In tube 1, 1.0 cc; 3, 0.8 cc; 5, 0.8 cc; 7, 0.5 cc; 8, 1.0 cc.

A few drops of pepsin are added to test-tube 1, afterward all test-tubes are corked, shaken up and put in the incubator for three hours (37° C.), after which the test-tubes are read off. The contents in tube 1 must remain clear, in tube 8 they must be cloudy. Then it must be ascertained at what dilution a noticeable clarification is effected. In order to obtain standard solutions for comparison *Solms* arbitrarily puts down that 1 cc of gastric juice contains 100 pepsin-units, which is just about sufficient to clarify the richin solution after three hours in the incubator for 1 cc of a 100 times diluted gastric juice. Normal gastric juice contains 100 to 200 pepsin units. In case of hyperacidity dilutions up to 1:10,000 must be employed.

6. **Renin and Reninogen.**—Reninogen is, like pepsinogen, a product of the gastric glands, and is converted by the action of hydrochloric acid into renin. Renin possesses the property of coagulating milk without the aid of the acids of the stomach, and, in fact, in the presence of a slightly acid or neutral reaction. The action of the ferment is absent when the reaction is slightly alkaline, but appears upon the addition of solutions of calcium salts. The action of the calcium salts is explained by the fact that they convert reninogen into renin.

Detection of Renin.—Ten cc of the filtered gastric contents are exactly neutralized with weak sodium hydrate solution (0.5 per cent.), and mixed with an equal quantity of neutral or amphoteric boiled milk. The mixture is placed in an incubator. When renin is present the casein will coagulate within ten to thirty minutes, and after longer staying will form a single coagulum (cheese). It must be determined each time after the coagulation that the reaction of the mixture is unchanged, since the casein may have been coagulated by acids which have developed late.

Detection of Reninogen.—To 2 cc of the filtered gastric contents are added an excess of sodium carbonate, 2 cc of a 3 per cent. calcium chloride solution, and 10 cc of milk, and the mixture placed in an incubator. If reninogen is present coagulation will gradually take place.

According to *Boas* the quantitative analysis for rennet depends upon the principle of how far the gastric juice can be diluted without losing its coagulating power of milk. The filtered gastric contents are first neutralized with sodium hydrate. One cc is taken up with the pipette and is diluted with ten times its volume of water, half of this is again diluted with an equal volume of water. We proceed in the same way and prepare (50 cc every time) dilutions of 1 in 10, 1 in 20, 1 in 40, 1 in 80, 1 in 320, etc. Into each test-tube that contains 5 cc of the thus diluted gastric juice is added 5 cc lukewarm, boiled milk and 2½ cc of a 1 per cent. solution of chlorcalcium. The test-tubes are shaken up a few times and put into the incubator or into a warm-water bath of 40° C. After fifteen to twenty minutes we observe at which lowest dilution coagulability is still seen. In normal secretion a dilution of 1:160 shows firm coagula, while in the next dilution of 1 in 320 only a fine flocculent precipitate is seen. In cases of hypersecretion we get positive results even in a dilution of 1 in 800.

The fermentation-tests for rennet and pepsin are to be made for diagnostical purposes only, in such gastric contents which contain no free hydrochloric acid.

Bile Pigment.—The tests for bile-pigment are made according to the methods of *Gmelin* or *Rosin*. (Cf. Chapter VII.)

8. **Blood**—The tests for blood are made by way of chemistry, the microscope and by the spectroscope. The simplest chemical reactions are:

(*a*) *Guaiacum Test* (*According to Weber*).—A few cc of glacial acetic acid are added to 10 cc of the gastric contents and shaken with the same amount of ether. After a sediment has formed a few cc of the etheric extract are poured off and mixed with 20 drops of resinous turpentine or hydrogen-superoxide. Then a fresh-made alcoholic guaiacum solution is added, drop by drop, while shaking. The solution must not be too concentrated; too much guaiacum mars the test, one must be guided by the color, which is to be brownish-yellow, but not dark-brown. The color changes to blue-violet if blood is present, but in the absence of blood-pigment to red-brown; after dilution with water the blue pigment can be extracted with chloroform.

(*b*) *The Aloin Test.*—The tincture of guaiacum is substituted by a fresh-made aloin solution (the tip of a knife of aloin in 10 cc alcohol). The color changes instantly or after some time to strawberry-red.

(*c*) *The Benzidin Test* (is to be used only when there are no rests of meat in the gastric contents). In a test-tube is put benzidin—a tip of a knife, 1 cc glacial acetic acid, 2 cc hydrogen-superoxide. In the other test-tube a few cc of the gastric contents are heated to the boiling-point, 3 to 5 drops from the second tube are poured into the first tube and shaken. If blood-pigment is present, the color becomes emerald-green or green-blue.

This test is extremely sensitive.

The *spectroscopical* test is made in the same way as the examination of the urine (cf. ibidem).

When the gastric contents contain free hydrochloric acid and a large quantity of organic acids, oxyhæmoglobin is converted into hæmatin chloride. The latter is only slightly soluble in water. Therefore under these conditions the spectroscopical examination may yield a negative result even when a large quantity of blood is present. It is well in such cases to treat the gastric contents, according to *Weber*, with a few cc of concentrated acetic acid, and shake thoroughly with ether. When blood is present the gastric contents assume a reddish-brown color, and show the spectrum of hæmatin chloride.

9. **Hydrogen sulphide** is easily recognized by its characteristic odor. It can, in addition, be detected by the following simple test:

A strip of paper, saturated with an alkaline solution of lead acetate, is fastened in a cork. The vessel containing the gastric contents is tightly closed with this cork so that the strip of paper is entirely within the vessel. When hydrogen sulphide is present the paper turns black.

Quantitative Chemical Examination of the Gastric Contents

1. **Estimation of Total Acidity.**—In the estimation of the total acidity all acid-reacting substances in the gastric contents come into consideration:

(*a*) Free and combined hydrochloric acid.

(*b*) Free and combined organic acids (lactic, butyric, and acetic acid).

(*c*) Acid phosphates.

The acidity is expressed by the quantity of decinormal alkali solution which must be added to 100 cc of the gastric contents in order to neutralize them.

Procedure.—To 10 cc of the filtered gastric contents in a small beaker, 1 or 2 drops of an alcoholic solution of phenolphthalein are added. Decinormal alkali solution is run into it from a *Mohr's* burette, with vigorous shaking, until the liquid assumes a distinct red color. The level of the liquid in the burette is noted before and after the titration. The amount of decinormal alkali solution used is determined by subtraction and multiplied by 10.

2. *Estimation of Free Hydrochloric Acid.*

(*a*) According to Minz.—According to this method, the gastric contents are treated with decinormal alkali solution until the reaction for free hydrochloric acid just disappears.

Procedure.—Ten cc of the filtered gastric contents are titrated in a beaker with decinormal alkali solution. At first the solution is added 1 cc at a time, and after the addition of each cc, *Günsburg's* reaction is carried out with a drop of the solution. The titration is proceeded with in this manner until *Günsburg's* reaction disappears. The approximate amount of decinormal alkali solution needed to neutralize the free hydrochloric acid, which is so obtained, is then rendered more accurate by adding to another 10 cc of the gastric contents, 1 cc less of the decinormal alkali solution than was previously used, and proceeding with the titration, adding the alkali 1 drop at a time. After every second drop, *Günsburg's* reaction is carried out.

If it is found, for example, that after the addition of 2.5 cc the reaction is still present, while after the addition of 2.6 cc it is absent, the amount of free acid is 2.6 × 10 = 26 cc decinormal alkali solution (calculated for 100 cc of gastric contents). Each cc of the decinormal alkali solution represents 0.00365 gramme of hydrochloric acid. The percentage in this case is, therefore, 0.00365 × 26 = 0.0949 per cent. This method gives reliable and, for practical purposes, thoroughly useful results.

(*b*) According to Toepfer.—According to this method the free hydrochloric acid is estimated, using a

0.5 per cent. alcoholic solution of dimethylamidoazobenzol as an indicator.

Procedure.—To 10 cc of the filtered gastric contents, 2 to 3 drops of the dimethylamidoazobenzol solution are added; to the now bright red liquid decinormal alkali solution is added from a burette until the red color of the fluid entirely disappears, giving place to the original yellow color. This method gives comparatively reliable results only when large amounts of hydrochloric acid and very small amounts of organic acids are present. Under the opposite conditions, it gives very inaccurate results, as the organic acids are included in the titration.

3. ***Estimation of Total Hydrochloric Acid According to Toepfer.***—The quantity of total hydrochloric acid is computed from its components, the free and combined hydrochloric acid.

The free hydrochloric acid is estimated, according to the above-described method, by titration with dimethylamidoazobenzol, combined in the following manner:

Ten cc of the filtered gastric contents are titrated with decinormal alkali solution, 3 to 4 drops of a 1 per cent. watery solution of alizarin sulphonate of sodium being added as an indicator, until the originally yellow liquid passes through red into a pure violet. Since alizarin reacts with all the factors of acidity except combined hydrochloric acid, the subtraction of the acidity found in this manner from the total acidity gives the amount of combined hydrochloric acid. Example: In the titration of 10 cc of filtered gastric contents with alizarin sulphonate of sodium, 4.5 cc of decinormal alkali solution are used—i.e., 45.0 cc to 100 cc. The total acidity was previously estimated, and amounted to 50.0 cc of the decinormal alkali solution. The acidity due to combined hydrochloric acid is, therefore, $50-45=5.0$. If this number is multiplied by 0.00365, the percentage of combined hydrochloric acid is obtained: 5 × 0.00365 = 0.018 per cent. Granted that the quantity of free hydrochloric acid (estimated by titration with dimethylamidoazobenzol as indicator) = 0.15 per cent., then the total hydrochloric acid amounts to 0.15 + 0.018 = 0.168 per cent. By subtracting the acidity due to free and combined hydrochloric acid from the total acidity, the acidity due to organic acids and acid phosphates can be determined.

4. **Estimation of Lactic Acid.**—According to *Leo*, 10 cc of the filtered gastric contents are boiled until the escaping steam no longer reddens a moistened strip of blue litmus-paper. The liquid, which has in this manner been freed from volatile acids, is then extracted with ether six times, 50 cc of ether being used each time. The ethereal extracts are poured together, and the ether distilled or driven off on a water-bath. The residue is taken up in a small quantity of water and titrated with decinormal alkali solution, 2 to 3 drops of phenophthalein being added as an indicator. Each cc of decinormal alkali solution used represents 0.009 gramme of lactic acid.

According to *Mehring* and *Cahn* lactic acid and the volatile fatty acids can be determined with the same portion of gastric contents. A measured quantity of the filtered gastric contents is distilled. The volatile acids which have gone over in the distillate are estimated by titration, while the residue is shaken repeatedly with ether. The lactic acid in the combined ethereal extracts is estimated in the same manner as in *Leo's* method.

Microscopical Examination of the Gastric Contents

For microscopical examination, the gastric contents are allowed to settle, a small portion of the precipitate withdrawn with a pipette, and specimens made in the usual manner.

Under normal conditions the microscopical examination of the gastric contents after *Ewald's* test-meal shows numerous starch-granules, isolated yeast-cells, epithelium from the oral cavity, a little mucus or particles of swallowed sputum. For diagnosis these constituents of the chymus are of no value, only the microscopical examination of the contents of the *empty* stomach furnishes material for diagnostical purposes. If HCl is present in the empty stomach, the following constituents are found during the microscopical examination:

1. Nuclei of leucocytes and epithelium (the protoplasm is digested).
2. Mucus of distinctly striate structure.
3. Spiral-cells, i.e., snakelike formations, originating from the myelin of the swallowed sputum under the influence of the HCl.

These three constituents are found with normal secretion and with hypersecretion, but no food-rests in the stomach contents. If, besides these constituents, food-rests are found in the empty stomach, a stagnation is present. Besides the food-rests such as starch-granules, muscle fibres, drops of fat, crystals of fatty acids, rests of vegetables, etc., there are found numerous sarcinæ or yeast-cells. The sarcina appears in the stomach contents in two different forms: 1. In form of bales. 2. In irregular lumps or in form of cubes. Characteristic for the sarcina is the cellulose reaction; it changes to red-violet after a chlor-zinc-iodine solution is added (chlor-zinc, 20.0; potassium iodine, 6.5; iodine, 1.3; water, 10.5). The yeast-fungi appear as oval, quite highly refracting, often pearl-necklacelike arranged cells, which are readily distinguished from the small starch-granules by adding a iodine-potassium-iodine solution (*Lugol's*); starch colors blue, yeast-fungi color yellow. In the absence of HCl and other free acids (achylia gastrica, gastritis simplex) mostly unchanged epithelia are found in the empty stomach and isolated leucocytes, sometimes amœbæ and infusoria. In malignant diseases of the stomach (tumors) also red blood-corpuscles and many pus-corpuscles are found.

Schizomycetes are only found when they are present in very large numbers and entirely obscure the microscopical field. Of the kinds of bacteria, *Boas*'s "faden (thread) bacilli" are quite frequently found in carcinoma of the stomach. They appear as long, slightly motile rods, lying usually at an angle to one another. They are, to be sure, not pathognomonic of carcinoma of the stomach, but they are found in nearly 75 per cent. of the cases. They are also present in cases in which there is stagnation of the gastric contents with the absence of free hydrochloric acid and production of lactic acid.

Crystalline bodies are comparatively rare in the gastric contents. The following have been described: Leucin and tyrocin crystals (in stagnation), triple phosphate crystals and crystals of magnesium phosphate (only in alkaline or neutral gastric secretions), and, very rarely, cholesterin crystals. For the identification of the crystals micro-chemical reactions are best used (cf. Microscopy of Urine).

CHAPTER VI

EXAMINATION OF THE FAECES

General Characteristics

1. ***Color.***—Under normal conditions, in the adult, hydrobilirubin is the characteristic fecal pigment: bilirubin is present normally only in the fæces of nursing children. The coloring of the fæces is not due to pigments alone, but is influenced by a great number of factors, of which, as a rule, the character of the food is the most important. Under a mixed diet the fæces are yellowish-brown in color, under a meat diet, dark to blackish-brown, and under an exclusive milk diet, orange to light yellow. Foods which have a peculiar color of their own may produce a characteristic coloration of the fæces. For example, following the liberal ingestion of chlorophyllic vegetables or of lettuce, the fæces may be stained green; following that of "blutwurst," blackish-brown; and that of cocoa, blackish-red. Black cherries and blackberries stain the fæces blackish-red; red wine and blueberries, reddish-brown with a tinge of green. Drugs also frequently cause a characteristic coloration of the fæces. The green coloration following the use of calomel is very well known. It is due to the conversion of bilirubin, within the intestinal tract, into biliverdin. Following the use of bismuth the fæces are colored black. The coloration is due to the reduction of bismuth subnitrate to black oxide of bismuth. Preparations of iron also fre-

quently produce a black coloration of the fæces, which is, however, limited to the surface.

In pathological conditions the pathological products of the intestinal wall may influence the color of the fæces more or less. Thus, the liberal admixture of mucus or pus may produce a grayish-white to yellowish-gray color. Blood, depending upon the quantity and upon the degree of alteration of the hæmoglobin, may produce a bright-red to pitch-black coloration. Bacteria may also produce a characteristic coloration of the fæces. It has been possible to cultivate from the stools of nursing infants and children a bacillus ("bacille de la diarrhée verte des enfants," *Lesage*) whose cultures contain a pigment which colors the fæces green. *Bacillus pyocyaneus* may also, under certain conditions, produce a green coloration of the fæces.

As the result of obstruction of the bile-duct (by catarrhal swelling, gall-stones, tumors, ascaris, etc.), the stools are clay-colored (acholous), and contain considerable fat. In cases of intestinal hyperperistalsis, with excessive diarrhœa, unaltered bile-pigment (biliverdin) may stain the fæces green.

2. **Consistency and Form.**—According to the consistency a distinction is made between firm or formed, thick or thin-pasty, and watery stools. Following a chiefly animal diet the stools are, as a rule, cylindrical and firm. Following a vegetable diet they are usually thick-pasty. The firm fæces have occasionally a "pencil form" (in stenosis or spasm of the large intestine), or the so-called "sheep manure" form. In the latter case, round balls the size of a hazel-nut are evacuated. Thin-pasty and watery stools are usually pathological. Following marked hemorrhage in the upper bowel or stomach the fæces have a black, tarry appearance.

3. **Odor.**—The odor of the fæces is, under normal con-

ditions, due principally to the presence of skatol, a product of the decomposition of albuminoid substances. Indol, which is produced at the same time, has a lesser influence upon the odor of the stools.

The odor of the fæces, therefore, depends upon the diet and the degree of decomposition in the intestinal tract. Following a meat diet, rich in albumin, the fecal odor is much more marked than following a vegetable diet, and in atonic conditions of the intestines it is stronger than when intestinal peristalsis is normal. Under an exclusive milk diet the odor is very slight, and therefore the normal stool of the nursing child is practically odorless. Every foul-smelling stool from a nursing child must, therefore, be considered pathological.

In acute and chronic diarrhœa the stools are often odorless. The characteristic rice-water stools of Asiatic cholera are also, as a rule, odorless. The evacuations in amœbic dysentery have a characteristic gluey odor. Acholous stools are of themselves nearly odorless. They possess a foul odor only when decomposition resulting from atony of the intestines accompanies the absence of bile. The stools are fetid and foul smelling in cases of ulcerating and decomposing carcinoma of the rectum.

4. ***Macroscopical Constituents.***—A superficial examination does not suffice, as a rule, for the detection of the macroscopical constituents of the fæces. For this purpose watery stools must be poured into shallow dishes, while thick-pasty and firm stools must first be carefully stirred with a glass rod in a large quantity of water. The macroscopical constituents are best collected by means of the fecal sieve suggested by *Boas*. This consists (Fig. 11) of two hemispheres, which are held together by means of a bayonet catch, and can easily be taken apart. The lower hemisphere contains an exceptionally fine sieve

(S), upon which the fæces are spread out. The upper hemisphere has a nozzle for a tube, by which it may be connected with any water-spout, and a chain with which to hang it from the spout. The water is carefully turned on, and a continuous fine stream allowed to flow over the fæces.

In the upper hemisphere is an opening (O), with a removable cap, through which a glass rod may be introduced, with which, during the washing, the fæces are stirred to a pasty mass. The water escapes through a pipe in the lower hemisphere. This procedure takes fifteen to thirty minutes, and only the coarser constituents of the fæces remain upon the sieve.

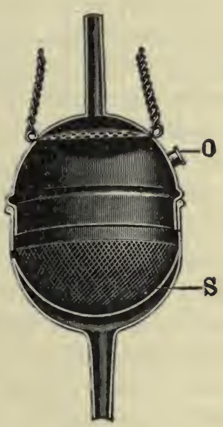

Fig. 11

The macroscopical constituents of the fæces especially to be noticed are:

1. Undigested food particles.
2. Pathological products of the intestinal wall.
3. Intestinal parasites.

4. Gall-stones and enteroliths.
5. Objects which have been accidentally swallowed.

Of the constituents of animal diet, normally only those which are indigestible, as cartilage and tendon, and pieces of bone which have been accidentally swallowed, are found in the fæces. The presence of pieces of muscle or connective tissue when meat has been properly prepared and not ingested in very great quantity is considered pathological.

Of the vegetable foods, white bread, potatoes, farinaceous foods, and juicy fruits (without the peels), leave no undigested portions which can be recognized macroscopically. Raw vegetables (cucumbers, lettuce, onions, radishes, asparagus, and string beans) and numerous fruits (cranberries, nuts, and currants) pass through the intestinal canal and appear in the fæces practically unchanged. Cooked fruits and vegetables are very much more easily digested; and, as a rule, only the poorly cooked and insufficiently masticated portion can be recognized in the fæces macroscopically. At any rate, no diagnostic conclusions can be drawn from the appearance in the fæces of undigested particles of vegetable matter.

Of the products of the intestinal wall which may be found in the stools, mucus is of particular importance. According to *Nothnagel*, every admixture of mucus with the stools should be considered as a deviation from the physiological. Mucus may be seen macroscopically in the fæces in varying form, consistency, and quantity.

In diseases of the lower portions of the bowel, mucus appears, in larger or smaller quantity, as a glassy substance, which is not mixed with the fæces. In membranous enteritis, shreds of false membrane and strips of mucus are present. When the mucus comes from the upper portion of the large intestine, it is thoroughly mixed with fecal matter (if the latter is pasty or fluid in consistency), or appears in small strips, just visible to the naked eye.

Admixtures of pus, which can be recognized macroscopically, come from the lower part of the intestinal tract, as pus coming from the upper portions undergoes such physical and chemical alterations that its macroscopical recognition is no longer possible.

Blood may be mixed with the stools in a fresh, coagulated, or decomposed condition. In the last instance the fæces have a tarry appearance. It is usually assumed that the darker the blood appearing in the fæces, the higher the location of the hemorrhage.

Particles of tumors (fragments of carcinoma, exfoliated intestinal polypi) can only be recognized by the aid of a careful histological examination, for macroscopically they may be confused with undigested pieces of meat.

Of the macroscopical parasites the most common are: Proglottides of tapeworms, *Ascaris lumbricoides*, *Anchylostoma duodenale*, *Oxyuris vermicularis*, *Trichocephalus dispar*, and, rarely, insects and their larvæ.

Enteroliths and gall-stones are distinguished usually from other constituents of the fæces by their form, consistency, and surface. They are, however, not infrequently confused by the patient, as well as the physician, with various other solid constituents of the stools; so that a careful microchemical examination alone renders a certain determination of the character of the object in question possible, in each individual case.

Foreign bodies, which are accidentally swallowed and reappear in the fæces, are of most varied character. Usually they pass through the intestinal tract unaltered, and are therefore easily recognized without further examination.

5. ***Quantity of the Faeces.***—The daily quantity of the fæces differs widely under normal conditions, and therefore no conclusions of diagnostic worth can be drawn from it. The amount of the fæces depends upon the quantity and character of the food and the condition of the digestive organs. Vegetable foods produce a much larger quantity of fæces than animal. In pathological conditions of the digestive tract, the quantity of the fæces may be markedly increased, due either to interference with absorption or to the admixture of pathological products of the intestinal wall, mucus, pus, blood.

Qualitative Chemical Examination of the Faeces

1. ***Reaction.***—Under normal conditions the fæces show no marked deviation from a neutral reaction. They are usually faintly alkaline or neutral; a faintly acid reaction appears only following an exclusively vegetable diet. The test is made in the usual manner with litmus-paper. Two strips of litmus-paper (red and blue) are moistened with distilled water, applied to the fæces, and the change of color is noticed on the clean side. The fæces must be thoroughly mixed before the examination, as it frequently happens that they are composed of constituents having various reactions, and that they react differently on the surface than in the deeper portions. In addition, the examination must be made as soon as possible after evacuation, as changes of reaction often occur very quickly. Hard stools must be thoroughly mixed with distilled water.

2. ***Mucin.***—When the entire quantity of fæces is to be examined for mucin, they are thoroughly mixed with water and an equal quantity of lime-water is added to them. The mixture is allowed to stand for a few hours, filtered, and the filtrate treated with acetic acid. If mucin is

present, a precipitate is thrown down, which is not soluble in an excess of acetic acid. However, to recognize the precipitate with certainty as mucin, the following facts must be established: (1) That it contains no phosphorus; (2) that after boiling a short time (ten to twenty minutes) with a 7.5 per cent. hydrochloric acid solution, it strongly reduces *Fehling's* solution.

To identify admixtures with the fæces which have a mucous appearance, as such, by means of the detection of mucin, they are dissolved in a weak sodium hydrate solution, and tested with acetic acid. This precipitate must also be tested for phosphorus and as to its reducing power after boiling with hydrochloric acid.

3. ***Fat.***—Fat frequently appears in the stools under normal conditions; it is composed, usually, of a mixture of neutral fat, fatty acids, and soaps (calcium and magnesium soaps).

The qualitative detection of fat in the fæces is very easy. They are mixed with a small quantity of ether, allowed to settle, a small portion of the ether withdrawn with a pipette, and a drop allowed to evaporate on a piece of filter-paper. A transparent spot, which cannot be washed out with water, remains. The fact that the stools contain fat can, however, have no diagnostic significance, since, as has been already mentioned, it is often normally present in quantities easy to detect, particularly following the liberal ingestion of fat. Occasionally, therefore, for diagnostic purposes, a quantitative estimation of the total fat must be made.

4. ***Blood.***—When blood in an undecomposed condition is mixed with the fæces, it can be easily recognized macroscopically; as a control, the microscopical detection of red blood-corpuscles or the spectroscopical detection of oxyhæmoglobin is sufficient. When, however, the blood-

pigment is altered, it can be detected only by chemical and spectroscopical means.

(*a*) *Chemical Detection According to Weber.*—A portion of the fæces is thoroughly mixed with sufficient 30 per cent. acetic acid solution to render it liquid, and extracted in a test-tube with ether. A portion of the ether, which, when blood is present, is brownish-red in color, is treated with twenty to thirty drops of old turpentine and ten drops of fresh tincture of guaiacum. On shaking, a blue-violet coloration appears. The blue pigment can, after the addition of water, be extracted with chloroform. The rest of the brownish-red ethereal extract may be used for—

(*b*) *The Benzidin Test.*—Put in a test-tube the tip of a small knifeful of benzidin, 1 cc of glacial acetic acid, and 2 cc of hydrogen superoxide and shake well. In another tube rub a pea-sized piece of fæces with a glass-rod in about 5 cc water and heat to the boiling-point. Pour a few drops of the latter solution in the first test-tube and shake. If blood is present, the solution changes to from green to blue-green. This extremely sensitive test is of positive diagnostical value in meat-free diet only.

(*c*) *The spectroscopical examination* can be made with the acid-ether-extract which has been obtained from *Weber's* test; this examination only gives a positive result, when the fæces contain larger quantities of blood; the extract then shows a distinctly brown-red color. At the same time are seen the characteristic four absorption-bands of hematin in acid solution: 1. In red. 2. In yellow. 3. Between yellow and green. 4. Between green and blue. As a rule only the first band (in red) shows distinctly.

5. ***Biliary Constituents.***

(*a*) *Bile-Pigments.*— Under normal conditions, the stools of the adult contain no unaltered bile-pigment:

bilirubin or biliverdin. The color of the normal fæces is due principally to the reduced bilirubin—hydrobilirubin (identical with urobilin).

Hydrobilirubin is detected, according to *Schmidt*, in the following manner: Fresh fæces (a piece the size of a hazel-nut) are thoroughly rubbed in a mortar with a concentrated watery solution of corrosive sublimate, and allowed to stand for several hours in a wide dish. Portions of the fæces containing hydrobilirubin are then deep red in color (due to the formation of hydrobilirubin-mercury), while those containing unaltered bilirubin are green.

According to *Schlesinger*, hydrobilirubin in the fæces is detected, as is urobilin in the urine, by means of an alcoholic solution of zinc acetate.

In addition to the above-mentioned test, the following reactions may, according to *Schmidt*, be used to detect unaltered bilirubin in the fæces.

1. *Gmelin's Test.*—A few drops of nitric acid, which contains nitrous acid, are placed in a porcelain dish, and a few drops of fæces, well mixed with water, are allowed to run into them. A play of colors is produced, composed of green, blue, violet, red, and yellow. The green color is characteristic of bilirubin. This test can also be carried out on a slide and observed microscopically.

2. *Huppert's Test.*—Twenty to thirty cc of fæces are mixed with sufficient water to render them thinly liquid, treated with an equal quantity of milk of lime, thoroughly shaken and filtered. The precipitate on the filter is washed with water and together with the filter is placed in a beaker, treated with a small quantity (5 to 10 cc) of alcohol slightly acidified with sulphuric acid, and carefully heated to the boiling-point. When bilirubin is present the liquid assumes a green color.

(*b*) *Biliary Acids.*—Normally, the biliary acids are absorbed from the fæces in the upper portion of the intestinal tract, so that their appearance in the stools must be considered as pathological. For the detection of biliary acids, a small quantity of the fæces is extracted with alcohol and filtered. The filtrate is distilled, to drive off the alcohol, and the residue taken up by water rendered faintly alkaline with soda. *Pettenkofer's* test is carried out with the watery solution; that is, the solution is treated with cane-sugar and a few drops of sulphuric acid. In the presence of biliary acids a red coloration is produced.

Quantitative Chemical Examination of the Faeces

1. Estimation of Dry Matter

A weighed portion of the fæces is first dried in the air on a water-bath. It is well to mix a small quantity of dilute sulphuric acid with neutral or alkaline fæces, in order that there be no loss of NH_3, which may be of importance in a subsequent estimation of nitrogen. The air-dried fæces are not yet free of water, and must therefore be further dried at a higher temperature, until a point of constant weight is reached. This procedure is difficult when the stools are rich in fat, and it is well, therefore, to evaporate stools containing a macroscopical quantity of fat with a weighed quantity of calcined sand. When this is not done, the air-dried fæces should be mixed with about ten times as much weighed sand. Stools not rich in fat are dried in an air drying-oven at 105° C.; while those rich in fat must remain about thirty to forty hours in a water drying-oven at 98° to 99° C. The fat must not be subjected to a higher temperature, as it melts and forms a coating over the moist mass, which hinders further drying. When the fæces are dried in an air-oven they are weighed every three hours, until a point of constant weight is reached. When they are dried in a water-oven they are first weighed after twenty-four to thirty hours, and then every six hours. Under a mixed diet the dry matter constitutes about 25 per cent. of the fæces; under a purely vegetable diet it is considerably less (10 to 15 per cent.).

2. Estimation of Total Nitrogen

The nitrogen of the fæces is usually estimated according to the method of *Kjeldahl.* This method is carried out in the following manner: 1 to 1.5 grammes (carefully weighed) of fæces, dried under the addition of dilute sulphuric acid, are treated in a *Kjeldahl* flask, with 20 cc of *Kjeldahl* sulphuric acid and a few drops of a concentrated copper sulphate solution, and allowed to stand six to twelve hours. The flask is then heated on a sand-bath, in a fume-chamber, until the liquid becomes colorless, or very faintly wine-yellow in color. Further details are carried out in the same manner as in the estimation of nitrogen in the urine.

3. Estimation of Fat

The fat in the fæces consists of a mixture of oleic, palmitic, and stearic acids and their salts (soaps), and glycerine ethers (neutral fats). The relative quantities of these components of the fæces vary greatly, and depend principally upon the character of the fats in the diet. It is the estimation of the total quantity of fat which is of clinical importance; a separate estimation of neutral fats, fatty acids, and soaps is undertaken only in special examinations, while the separate estimation of oleic, stearic, and palmitic acids has absolutely no practical value.

Estimation of the Total Fat of the Faeces.—The simplest method is extraction with ether. Only the neutral fats and free fatty acids are soluble in ether. The soaps must, therefore, be decomposed before the extraction.

Three to four grammes (exactly weighed) of the dried pulverized fæces are mixed with a small quantity of 1 per cent. HCl acid alcohol, and dried on a water-bath, by which procedure the soaps are decomposed. The dry residue is placed in the chamber of a *Soxhlet* apparatus (the dish being thoroughly cleaned with pieces of filter-paper, which are also placed in the chamber). The extraction is continued twelve to twenty-four hours. After the extraction is completed, the ether, which is collected in a light, previously weighed flask, is distilled, the last trace being driven off by a stream of air, and the residue dried for some hours at 80° C., or for a short time at 105° C., and then weighed.

The disadvantage of this method is, that besides the neutral fats, fatty acids, and soaps, other substances soluble in ether—as cholesterin, lecithin, cholic acid, and pigments—are included in the estimation. The quantity of these substances in the ethereal extracts is, however, comparatively small, so that for the usual clinical estimation of fat it may be disregarded.

4. Estimation of Carbohydrates

(*a*) ***Indirect Estimation of Total Carbohydrates.***

According to this method the carbohydrates are estimated as nitrogen-free extracts, the values of the albumin, fat, and ash being subtracted from the dried fecal matter. It is self-evident that this method gives comparatively inexact and practically useless results: first, because the estimated residue contains other substances in addition to carbohydrates (vegetable acids, pigments, etc.); secondly, because no judgment of the extent of digestion can be formed from the total quantity of carbohydrates.

Such a judgment can only be rendered possible by separating the practically indigestible cellulose from the readily soluble starch. As, however, we possess no exact and simple method for the quantitative estimation of cellulose, a direct estimation of starch is undertaken in order to judge of the extent of the digestion of the carbohydrates.

(*b*) ***Direct Estimation of Starch According to Liebermann and Allihn.***

The principle of this procedure is that starch is converted into grape-sugar by boiling with hydrochloric acid, the sugar solution boiled with *Fehling's* solution, and the precipitated copper oxide reduced by hydrogen to metallic copper. From the quantity of copper the quantity of grape-sugar is determined, and from it the starch is calculated.

When the fæces contain a liberal admixture of mucus, it must, as far as possible, be removed with forceps, as mucin, when boiled with hydrochloric acid forms a copper reducing substance.

Three to five grammes of dried, pulverized, and exactly weighed fæces are treated in a flask with 100 cc of a 2 per cent. solution of hydrochloric acid, and boiled on a sand-bath for an hour and a half, using a back-flow condenser; the liquid is then neutralized with sodium hydrate, and filtered through an asbestos filter, by means of an exhaust-pump, into a 500 cc flask, and the residue washed with hot water until the filtrate amounts to 500 cc. Thirty cc of a 7 per cent. solution of copper sulphate (*Fehling's* solution No. 1), 30 cc of an alkaline solution of Rochelle salts (*Fehling's* solution No. 2), and 60 cc of water are placed in a beaker or porcelain dish and heated to the boiling-point. To the boiling liquid 25 cc of the sugar solution are added from a pipette, the liquid boiled three minutes, and the precipitated copper oxide collected on a filter.

For filtering, an asbestos filter-tube is used. The tube must be filled with long-fibred, soft asbestos; to prevent particles of asbestos from being washed through during the infiltration, a small plug of glass wool is placed at the conical end of the tube underneath the asbestos. The filtration is best accomplished by means of an exhaust-pump. The tube is dried at 100° C., and weighed before using. The copper oxide collected on the asbestos filter is first washed with cold water, then with alcohol and ether, and finally dried for fifteen minutes in a drying-oven at 100° C. A stream of pure, dry hydrogen gas is now, under slight heating, allowed to flow from a *Kipp's* hydrogen generator through the dry tube. As soon as the precipitate has assumed the characteristic copper color, and the tube is thoroughly dry, the heating is stopped, the tube is allowed to cool in the stream of hydrogen, and weighed. From the amount of copper oxide found the amount of grape-sugar is calculated.

(*c*) *Fermentation Test According to Schmidt.*

This test renders possible the detection and approximate quantitative estimation of the carbohydrates, which are easily acted upon by the digestive juices, and is therefore, especially as its performance is very simple, to be recommended as a method for estimating the efficiency of the digestive apparatus.

The principle of this method is that the dissolved carbohydrates, as well as those starches which lie free and are easily acted upon (enclosed in thin cellulose capsules), are inverted by the diastase which is always present in the fæces, and are then fermented by the intestinal bacteria, with the production of gas. The test is carried out in the following manner: 5 grammes of fæces are placed in the vessel of a *Schmidt's* fermentation apparatus (Fig. 12), well mixed with water, and the vessel closed with the rubber stopper, care being taken to exclude air-bubbles. Tube *b* is also filled with water, without air-bubbles and closed with the smaller rubber stopper. The entire apparatus is then placed in an incubator (37° C.) for twenty-four hours. The gas which is developed by the fermentation forces a portion of the water from tube *b* into tube *c*.

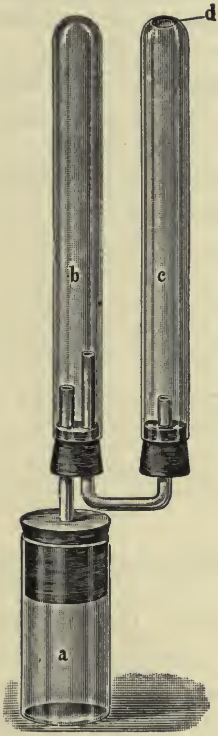

FIG. 12

The air in tube *c* escapes through the opening *d*. The quantity of gas produced, which corresponds to the quantity of fermentable carbohydrates, is judged from the height of the water in tube *c*. For diagnostic purposes only a positive result of the test is of value, as under pathological conditions the test may be negative even when sugar and starch are present.

According to *Schmidt*, intestinal dyspepsia may be diagnosed when, following a test-diet suggested by himself and *Strassburger*, enough gas is formed in twenty-four hours to fill tube *c* at least one-fourth full of water.

The test-diet consists of—

1.5 litres of milk
3½ eggs.
Gruel from 80 grammes of oatmeal.
100 grammes of zwieback (rusk).
20 grammes of sugar.
20 grammes of butter.
125 grammes of beef } raw.
190 grammes of potato } raw.

Examination of Gall-Stones and Biliary Concretions

General Characteristics

Gall-stones are usually pale yellow, or, more rarely, brownish-red in color. Stones of pure cholesterin are nearly colorless, and show a distinctly crystalline character. Their size varies greatly, from that of a pin's head to that of a walnut. They vary in hardness, though, as a rule, they are much softer and lighter than typical enteroliths.

On cross-section, gall-stones show not infrequently a distinct nucleus and a marked concentric stratification.

Naunyn divides gall-stones, according to their chemical characteristics, into the following groups:

1. Pure cholesterin stones with smooth or warty surface: on section white, and of crystalline structure.
2. Stratified cholesterin stones: colored and stratified.
3. Ordinary gall-stones: stratified, colored, but not crystalline.
4. Mixed bilirubin-calcium stones: stratified and colored, the nucleus consisting usually of cholesterin.
5. Pure bilirubin-calcium stones: dark brownish-red in color, the principal constituents being combinations of calcium with the biliary pigments—bilirubin, biliverdin, bilifuscin, and biliprasin, cholesterin being present in very small quantity, or not at all.
6. Amorphous cholesterin stones, conglomerate stones, and casts of the biliary passages, which are very rare.

Chemical Examination

As gall-stones and biliary concretions are combinations of calcium with biliary pigments, it is necessary for their identification as gall-stones, in cases in which the nature of the stones is not known, to detect chemically these principal constituents. For this purpose the following procedure is carried out: A stone is pulverized and boiled in water. By this means any traces of biliary acids which may be present are removed. The residue is then extracted with a warm mixture of equal parts alcohol and ether. The cholesterin is dissolved; the residue (1) contains the bile-pigments, which are combined with calcium, and the inorganic salts, which are insoluble in water. For the detection of cholesterin the alcohol and ether solution is separated from the residue by centrifugalization, and is allowed to evaporate. When cholesterin is present it forms large, very thin, characteristically placed, colorless, rhomboid plates; more rarely, it forms needles with a silky lustre.

For the identification of cholesterin the following reactions are used:

1. Concentrated sulphuric acid is allowed to run into the cholesterin on a slide, the crystals then dissolve at the edges and assume a carmine color; if *Lugol's* solution is added, a blue, red, green, and violet play of colors is seen.

2. A small quantity of perfectly dry cholesterin is dissolved in glacial acetic acid, and a few drops of concentrated sulphuric acid are added; a violet coloration is produced, which very quickly becomes green. The test succeeds only when the cholesterin is absolutely dry.

For the detection of bilirubin-calcium the residue (1) is covered with hydrochloric acid (when calcium carbonate is present foam is produced), and heated. The biliary pigments are by this means freed from their union with calcium. After cooling, the bilirubin is extracted with chloroform. The chloroform-extract may then either be allowed to crystallize, or may be used in carrying out *Gmelin's* test.

Fecal Concretions, Enteroliths, and Pancreatic Stones

By fecal concretions or coproliths are meant stony bodies, which are composed of hardened fecal matter. They are formed, as a rule, in those places in the large intestine at which stagnation of fecal matter can most easily take place; for example, at the flexures, or in the appendix vermiformis. Coproliths may reach such size and compactness that they cause complete intestinal obstruction. True intestinal stones (enteroliths) are much smaller than fecal concretions, and have in their entire character a much closer resemblance to other kinds of stones (urinary and biliary calculi). They consist usually of a nucleus of organic matter (fruit-pips, blood-clots, particles of fæces, etc.), about which layers of salts (usually earthy or triple phosphates) have been deposited. A distinction is made between the following forms of enteroliths:

1. **Typical Enteroliths.**—These are round, heavy, stone-hard, concentrically stratified, and contain a foreign body, the nucleus of the concrement.

2. **Light Stones.**—These are composed principally of undigested vegetable food particles, encrusted with phosphates. They are not stratified, and have no distinct nucleus. To this group belong the so-called "oatmeal-stones," which may form after the liberal and prolonged ingestion of oatmeal.

3. **Stones Composed of Drugs which have been Taken.**—Such stones consist principally of insoluble, or difficultly soluble drugs, which were taken in powder form. For example, salol, magnesia, calcium carbonate, etc.

4. **Intestinal Gravel.**—This consists of small, hard granules, which are usually composed of organic matter, calcium carbonate, and magnesium phosphate.

5. **Pancreatic Stones.**—These are very rarely found in the fæces. They are crumbly, and have a rough surface. They are readily soluble in chloroform, and on heating give off an aromatic odor. They are usually composed of calcium carbonate and phosphate. In the few cases reported in literature cholesterin and bile-pigments were detected.

For examination, the calculi are sawn through, and a small piece pulverized and tested by burning on a platinum spatula. If most of the powder burns up, the calculus consists principally of organic substances. In such cases microscopical examination will, in the majority of instances, disclose the composition of the calculus.

If, on the contrary, the calculus merely turns black on burning, leaving considerable residue, it is composed principally of inorganic substances. A qualitative analysis of these substances is carried out in the following manner: A portion of the pulverized stone is treated in a test-tube with dilute hydrochloric acid, and slightly heated. If gas develops on the addition of the hydrochloric acid, carbonates are present. The portions insoluble in hydrochloric acid consist principally of sand or of organic matter, and must be examined microscopically, the hydrochloric acid solution being separated from the residue either by filtration or centrifugalization. The fluid may contain phosphates (of calcium magnesium), calcium oxalate, ammonia, and traces of albuminoid substances. The detection of these constituents is accomplished in the same manner as in the examination of urinary calculi (q.v.).

Microscopical Examination of the Faeces

Fluid or thin, pasty stools are poured into a shallow dish, and when they are uniform in consistency a small portion is taken and spread between a cover-glass and slide. Any macroscopical objects which attract attention must be examined separately. Very thin stools are allowed to settle, or are centrifugalized, and the sediment examined. Formed stools are rubbed in a glass mortar with water or physiological salt solution. During the microscopical examination principally food particles (the great majority of which are of vegetable origin), bacteria, and crystalline bodies in small number are found. Under pathological conditions, pathological products of the intestinal wall and animal parasites may be present.

Of the food particles only those will be considered here whose presence in the stools may be of diagnostic significance. Among these are included:

1. ***Muscle-Fibres.***—These are in the stools nearly always heavily stained by bile-pigments, and are therefore easy to find. They are divided by *Schmidt*, according to their form and structure, into three groups:

(*a*) *Large.*—Distinctly striated pieces with sharp corners and outline.

(*b*) *Medium.*—Rectangles with rounded corners, whose striæ are still visible.

(*c*) *Small.*—Polygonal or round flakes, mostly homogeneous and with indistinct striæ.

The presence in the stools, following the limited ingestion of meat, of numerous muscle-fibres indicates a disturbance of the function of the small intestine, probably of the pancreatic digestion. When the food has been insufficiently masticated, shreddy objects, consisting principally of half-digested meat particles, may be frequently seen macroscopically in the stools of healthy persons.

2. ***Shreds of connective tissue*** are frequently seen during the macroscopical examination of the fæces. On microscopical examination they show a thready structure with delicate, often scarcely recognizable, fibrillation. In certain portions the interwoven elastic fibres can be distinctly seen. Upon the addition of acetic acid the structure of the connective tissue disappears, while the elastic fibres become more distinct.

The presence of much connective tissue in the stools, following the limited ingestion of meat (100 grammes), points toward disturbance of the gastric digestion, since the gastric juice alone is able to dissolve raw or incompletely cooked connective tissue. Following the ingestion of smoked meats the presence of connective tissue in the stools may be considered normal, as such raw connective tissue is digested with the greatest difficulty.

3. ***Occasional starch granules*** appear even in normal stools. Their marked increase indicates disturbance in the small intestine.

4. ***Fat.***—Fat is present in small quantity in all stools, and appears in the form of drops, flakes (neutral fat), or crystals (fatty acids, soaps). Fatty acids are distinguished from soaps by the fact that they melt when heated, while soaps remain unchanged. In addition, fatty acids dissolve readily in ether, while soaps must first be decomposed by acids. Upon the addition of a saturated alcoholic solution of Sudan III., neutral fat assumes an orange to blood-red color, while fatty acids and soaps remain colorless. Fat is increased in the stools in all diseased conditions in which there is an interference with its absorption from the food (affections of the intestinal mucosa, interference with biliary secretion, etc.).

In addition to the above-mentioned fatty acid crystals, the following crystalline bodies may be seen during the microscopical examination of the fæces: Triple phosphate ("coffin-lid"), neutral calcium phosphate, magnesium phosphate, calcium oxalate ("envelope"), calcium carbonate, calcium sulphate, cholesterin, and *Charcot-Leyden* crystals (in helminthiasis and enteritis membranacea).

The Pathological Products of the Intestinal Wall Especially to be Considered are:

1. ***Mucus.***—This appears microscopically as a structureless, transparent mass, in which epithelial cells, pus-corpuscles, crystals, or food particles are frequently embedded. Upon the addition of acetic acid (the mucus fleck should be thoroughly mixed with the reagent), the basic substance assumes a striated appearance. The pres-

ence of mucus in the fæces nearly always indicates a pathological condition of the intestinal mucosa.

2. **Epithelium**.—Squamous cells are very rarely present in the stools (diseases of the rectum); cylindrical cells, however, are more frequent. They rarely appear unchanged, but frequently in the so-called "verschollter" form (desquamated lumps), or in a half-digested condition. The presence of small mucus shreds, containing only half-digested epithelium, indicates inflammation of the small intestine. Desquamated epithelium comes usually from the large intestine. The presence of a large amount of epithelium in the stools indicates usually a catarrhal inflammation of the intestinal mucosa.

3. **Pus-Corpuscles**.—Leucocytes in small numbers are found in every mucus fleck. The appearance of a great number of pus-corpuscles indicates an ulcerative process in the intestines.

Red blood-corpuscles appear in the stools in unaltered condition only when the blood comes from the lower portions of the intestines, and has remained in them but a short time. If the blood comes from the upper portions of the intestines, the so-called "shadow corpuscles" may be occasionally found. As a rule, however, red blood-corpuscles can no longer be detected.

Intestinal Parasites and Their Eggs

1. **Amoebae**.—According to *Quincke* and *Roos*, three kinds of amœbæ are parasitic in man: *Amœba vulgaris*, *mitis*, and *coli* (dysentery). Recently the first two have been considered as one. The *Amœba coli* alone is accredited with pathological significance (Fig. 13). It is 10 to 15 millimetres in length, and is very motile. It contains, in addition to bacteria and ingesta, red blood-corpuscles, which is never the case with the *Amœba vulgaris* or *mitis*.

Amœba coli is considered by most authors as the exciting cause of amœbic dysentery. Its encysted form has a simple contour, while the encysted forms of the other two varieties have a double contour.

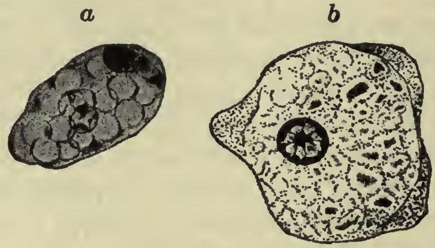

FIG. 13.—Amœba Coli (*a*, according to *Roemer*; *b*, according to *Doflein*)

The examination of the fæces for amœbæ must be made as soon as possible after evacuation, as these parasites resist cooling very feebly, and disappear rapidly. When the fæces are not perfectly fresh, only the encysted forms are to be found.

2. ***Infusoria*** (Fig. 14).—These are enclosed in a hard capsule, the surface of which is covered with flagella or cilia. In the fæces are found: *Cercomonas intestinalis*, *Trichomonas intestinalis*, and *Balantidium coli.* The last only is accredited with pathological significance. It

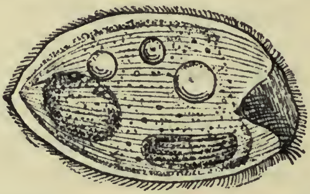

FIG. 14.—Balantidium Coli (according to *Leuckart*)

is not infrequently found in intestinal ulcerations. Whether this parasite enters the ulcerated mucosa secondarily, or is the cause of the ulcerative process, is not yet proved. The majority of authors doubt whether it is pathogenic for man.

3. ***Tapeworms* (*Cestodes*).**

(*a*) *Tænia Solium* (Fig. 15).—The cysticercus lives in swine. The worm is 2 to 3 millimetres long. Its scolex is unpigmented, has four suckers and a rostellum, which carries a double crown composed of twenty-six hooklets. The ripe segments (proglottides) are rather long when shed. The uterus has but seven to ten branches. The eggs are usually round (rarely oval), and enclosed in a thick shell, in which a distinct radial striation is seen. Not infrequently the hooklets of the embryo are visible within the egg.

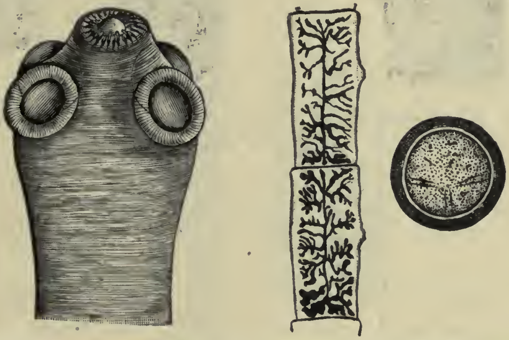

FIG. 15.—Tænia Solium: Scolex, Proglottides, Egg. (After *v. Jaksch.*)

(*b*) *Tænia Saginata* (*mediocanellata*) (Fig. 16).—The cysticercus lives in the muscles of the ox. The worm is 4 to 8 millimetres long. Its scolex has no rostellum and no crown of hooklets; it has four pigmented suckers. The uterus has twenty to thirty branches. These are best seen when the ripe segment is squeezed between two slides.

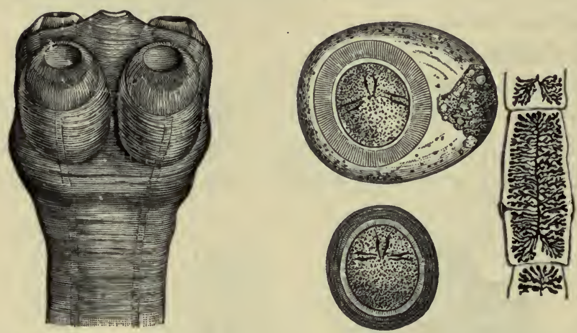

FIG. 16.—Tænia Saginata: Scolex, Eggs, Proglottides. (After *v. Jaksch.*)

The eggs are somewhat larger than those of the *Tænia solium*, but are in other respects hard to distinguish from them.

(*c*) *Bothriocephalus Latus* (Fig. 17).—The cysticercus lives in salt- and fresh-water fish. The worm is 6 to 8 millimetres long. Its long scolex with its long neck is

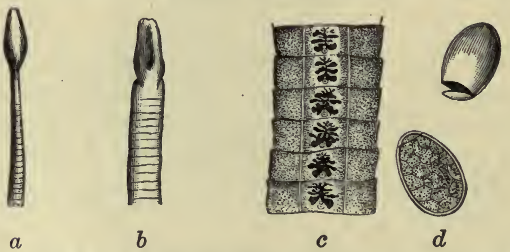

FIG. 17.—Scolex of Bothriocephalus Latus. *a*, Seen from above; *b*, from the side; *c*, proglottides; *d*, eggs. (After *v. Jaksch.*)

flattened out, and has two elongated suckers. The eggs are oval, and have a lid at one end. When the embryo is discharged the lid is lifted. The ripe segments are quadrilateral, and show a rosette marking in the centre, due to the brown egg-filled uterus.

The following tæniæ are more rarely seen: *Tænia nana*, *Tænia flavopunctata*, and *Tænia cucumerina*. *Tænia nana* is common in Italy and Egypt.

4. ***Round Worms (Nematodes).***

(*a*) *Oxyuris Vermicularis* (Fig. 18).—The eggs of this worm are swallowed and pass into the fæces, in which the worm completes its development. The male is 4 millimetres, the female 10 millimetres long. The eggs have a double contour, and are usually filled with a coarsely granular substance. Occasionally an egg is seen

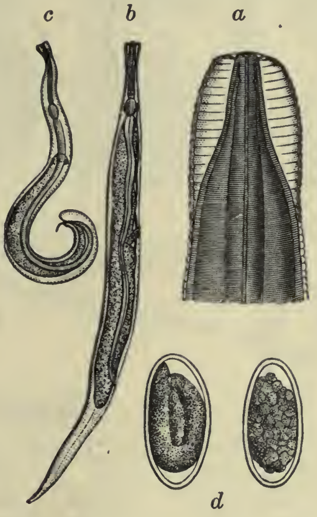

FIG. 18.—Oxyuris Vermicularis. *a*, Scolex; *b*, female; *c*, male worm; *d*, eggs. (After *v. Jaksch.*)

containing an embryo, in which the intestinal canal can be indistinctly seen.

(*b*) *Ascaris Lumbricoides* (*round worm*) (Fig. 19).—This worm is cylindrical and comparatively long—20 to 40 centimetres. The eggs are round or oval, yellowish-brown in color, and enclosed in an albuminoid capsule.

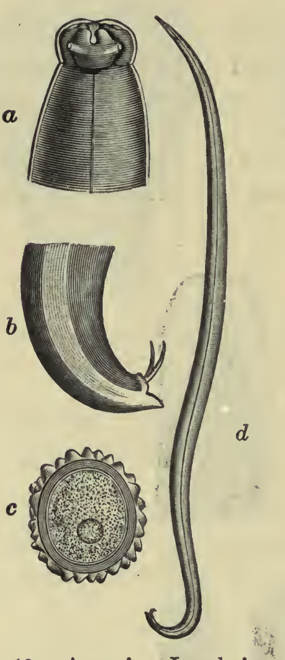

Fig. 19.—Ascaris Lumbricoides. *a*, Scolex; *b*, caudal extremity of the male worm; *c*, egg; *d*, male worm.

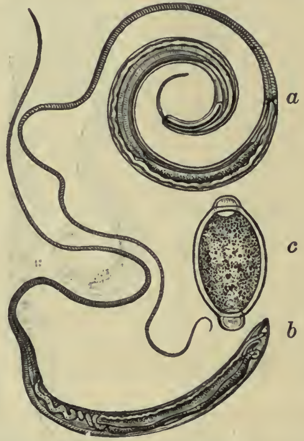

Fig. 20.—Trichocephalus Dispar. *a*, Male; *b*, female worm; *c*, egg.

(After *v. Jaksch.*)

(*c*) *Trichocephalus Dispar* (*whip-worm*) (Fig. 20).—This is usually considered as a harmless intestinal parasite; recently, however, *Metschnikoff* has accredited to it a significance in inflammation of the appendix vermiformis. The worm is about 4 centimetres in length. The eggs are easily recognized by the lid which they have at either end. They have a double contour, are brownish in color, and are filled with a granular substance.

(*d*) *Anchylostomum Duodenale* (Fig. 21).—As a rule, only the eggs are found in the fæces, since the worms themselves are so deeply and firmly embedded in the intestinal wall (small intestine) that they are not evacuated with the stools. The eggs have a single contour, are oval, and contain all stages of development of the embryo side by side. The male is 10 millimetres long, and has two

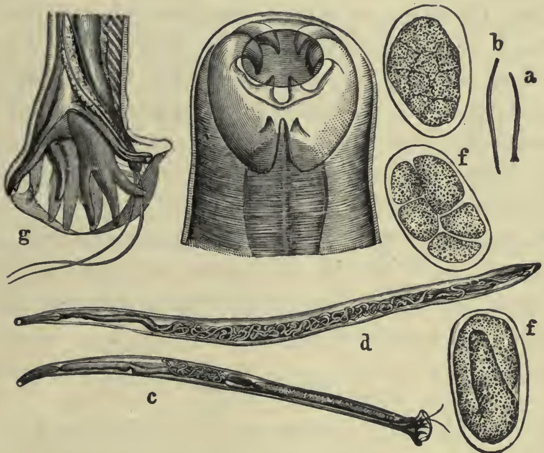

FIG. 21.—Anchylostoma Duodenale. *a*, Male worm (natural size); *b*, female worm (natural size); *c*, male worm (slightly magnified); *d*, female worm (slightly magnified); *e*, scolex; *f*, eggs; *g*, caudal extremity of the male. (After *v. Jaksch.*)

spicules at its caudal extremity. The female is pointed at the caudal extremity, and is 12 to 18 millimetres long.

Bacteriological Examination of the Faeces

The fæces possess normally a very luxuriant bacterial flora, the many varieties of which can be distinctly seen in specimens stained with dilute carbol-fuchsin and according to *Gram*. The bacteria present in greatest number are those belonging to the group of *Bacterium coli*. In addition, *Bacillus aerogenes*, varieties of subtilis and proteus, *B. fæcalis alkaligenes*, *B. fluorescens*, different varieties of cocci, fungi, and yeast-cells, are present. In cultures from the fæces only a very small per cent. of these micro-organisms (about 10 per cent.) develop. Upon the usual culture media bacteria of the coli group grow in overwhelming majority.

The most important pathogenic bacteria found in the fæces are typhoid, cholera, dysentery, and tubercle bacilli, more rarely strepto- and staphylococci, anthrax bacilli, plague bacilli, and *B. pyocyaneus*.

Typhoid Bacilli

The detection of typhoid bacilli in the fæces is still attended with considerable difficulty, and may not succeed even in cases which manifest themselves clinically, as undoubted typhoid. Often only repeated and laborious attempts succeed. Attempts made with the typical diarrhœal evacuations are the most likely to be successful, either because the bacteria are discharged in greater numbers or are more evenly distributed than in formed stools. In the latter they are frequently present in isolated spots only, in which case the presence of any bacteria at all in the material used for inoculation is more or less a matter of chance.

Characteristics of Typhoid Bacilli.—*Morphological and Staining Characteristics.*—The typhoid bacillus is a short rod which stains easily with dilute aniline dyes, and is decolorized by *Gram.* In hanging drops, typhoid bacilli, when grown on suitable culture media, are very motile.

Growth on the Usual Culture Media.—Typhoid bacilli grow upon all the usual culture media, and best at body-temperature.

Upon agar they develop small, moist, grayish-white colonies, which, when held against the light, show a bluish iridescence. They are more delicate, smaller, and more transparent than those of the *B. coli communis.*

On gelatine the surface colonies have usually a characteristic appearance; they appear delicate, iridescent, with jagged or wavy margins, and are traversed by numerous branching ridges resembling the ribs of a grape-leaf (grape-leaf form). This growth is, however, in no wise typical of typhoid bacilli alone, for there are varieties of coli whose colonies present the same, or a very similar, appearance. Typhoid bacilli do not liquefy gelatine.

On potato they develop a fine, colorless coating, invisible to the naked eye. There are, however, varieties of potato upon which, especially in the presence of an alkaline reaction, a gray, slimy coating is produced.

Bouillon is evenly clouded.

Growth of Typhoid Bacilli on Special Culture Media.—In the endeavor to simplify the isolation of typhoid bacilli from mixtures of bacteria, especially in attempts to cultivate from the fæces, a number of culture media have been suggested, upon which typhoid bacilli show conspicuous differences in their growth from other bacteria, particularly from those of the coli group. Only the two culture media most frequently used will be mentioned here: the *Conradi-*

Drigalski litmus-lactose-agar, and the *Piorkowski* urine-gelatine (for the preparation of these culture media, cf. Chapter XII).

Upon the *Conradi-Drigalski* culture medium typhoid bacilli produce, after fourteen to twenty-four hours' growth at 37° C., small glassy colonies with single contour, resembling dew-drops, and bluish in color, with a tinge of violet. Rarely, however, the larger colonies have a more clouded appearance. The colonies of *B. coli* are larger than those of typhoid bacilli, and are usually brilliant red and non-transparent. "Many colonies are only bright red, and not so cloudy; other varieties of coli produce larger colonies of waxy appearance, which are surrounded by a red-stained area."

It must, however, be mentioned that there are other bacteria whose growth does not change the color of this culture medium. Their colonies are frequently distinguished, however, from those of typhoid bacilli by their size, distinctly double contour, and their dull, dry surface. Among these are *B. fæcalis alkaligenes*, and bacteria of the subtilis, proteus, and fluorescens groups. The colonies of streptococci, which in attempts at cultivation from the fæces often develop in great numbers on this culture medium, also exactly resemble in color the colonies of typhoid bacilli. They are, however, very much smaller than these.

In *endos fuchsinagar* after having grown for twelve hours at 37° the coli-colonies become gradually red, starting from the centre; after twenty-four hours they are entirely red, round with prominent edges. The typhoid bacilli on the other hand are forming colorless round colonies with thin edges. After more than twenty-four hours the coli-colonies are dark-red, while the typhoid-colonies—now twice the size of the coli-colonies—remain colorless or are of a faint reddish color. The colonies of the bacteria which grow blue in the *Conradi-Drigalski* culture medium look like typhoid-colonies in fuchsin-agar.

This culture medium offers advantages against the litmus-lactose-agar, because it is prepared much easier and one can work in artificial light, while the blue colonies on the *Drigalski*-plates can be recognized in daylight only. A great disadvantage is the fact that, in endo-agar with many acid-formers present, the culture medium becomes diffusely red, which renders it impossible to recognize the colorless typhoid-colonies.

Thus the diagnosis cannot be made from the appearance of the colony alone, if fuchsin-agar and *Conradi-Drigalski* culture medium have been used; the suspicious looking bacteria have to be tested as to their morphological and biological properties in the same way as they would have been cultivated in ordinary agar. The fuchsin-agar and the litmus-lactose-agar offer the advantage over the agar, that they make it easier to detect the suspicious looking colonies.

Endo-Agar must be kept in the dark, as it otherwise gradually becomes red. The *Litmus lactose agar*, prepared according to the methods of *Conradi* and *Drigalski*, cannot be kept for any length of time, as in old culture media the difference between typhoid-colonies and coli-colonies does not appear distinct enough. To overcome this disadvantage the culture media have to be kept without litmus-solution. Crystal violet and sugar of milk and the substances are added only shortly before use (cf. Chapter XII).

Malachite Green Agar.—This culture medium, according to *Loeffler* (cf. Chapter XII), contains malachite-green so concentrated that it stops the growth of coli bacteria almost entirely, but hardly influences the growth of typhoid bacilli. Thus in the fæces coli bacilli do not grow at all, or very scarcely in this culture medium.

After twenty-four hours the typhoid-colonies appear in transmitted light delicate and transparent and are macroscopically hardly visible (about the size of a sand grain), the coli-colonies are thicker, non-transparent, of a whitish-cloudy appearance.

For cultures, taken from the fæces, *Loeffler* recommends to add 3 per cent. of sterile ox-gall to the malachite-green. The added malachite-green then must be 1.9 per cent. of a 0.2 per cent. pure solution of malachite-green-crystals.

Biological Characteristics of Typhoid Bacilli Important in Differential Diagnosis.—Typhoid bacilli do not coagulate milk; coli bacilli, however, do as a rule, after twenty-four to forty-eight hours.

B. fæcalis alkaligenes, dysentery bacilli, and paratyphoid bacilli also do not coagulate milk.

The growth of typhoid bacilli in litmus-whey (cf. Chapter XII) produces, after twenty-four hours, a small amount of acid, under 3 per cent., while the growth of coli bacteria produces more than 7 per cent. decinormal acid. Typhoid tubes show, therefore, only a slight reddish tinge, while coli tubes are bright red. Litmus-whey, inoculated with typhoid bacilli, remains perfectly clear, while that inoculated with coli bacilli becomes evenly clouded. *B. fæcalis alkaligenes*, by its formation of alkali, turns litmus-whey blue. Dysentery bacilli and type A of the paratyphoid bacilli act like typhoid bacilli, while type B produces at first a small amount of acid, but after a few days' growth alkali.

Growth in Barsiekow's Culture Medium (cf. Chapter XII).—In *Barsiekow's* nutrose-sodium chloride solution, containing 1 per cent. grape-sugar, typhoid bacilli and coli bacilli produce considerable acid and cause coagulation, while dysentery bacilli, at least during the first few days, produce very little acid and do not cause coagulation. This solution can, therefore, be used to distinguish between typhoid and dysentery bacilli. If, instead of grape-sugar, 1 per cent. milk-sugar is used, typhoid and coli bacilli may be differentiated by means of this culture medium, since typhoid and dysentery bacilli act in the same manner—i.e., they both leave the solution unchanged, while coli bacilli produce acid and cause coagulation. If *Barsiekow's* solution contains 1 per cent. grape-sugar and 1 per cent. milk-sugar, after twenty-four hours' growth—

Dysentery tubes show acid formation, but no coagulation.
Typhoid tubes show acid formation and clouding due to slight coagulation.
Coli tubes show acid formation and complete coagulation.

If the medium containing 1 per cent. grape-sugar is poured into fermentation-flasks, at the end of thirty-six hours the following conditions are present:

Dysentery tubes show acid formation.
Typhoid tubes show acid formation and coagulation.
Coli tubes show acid formation, coagulation, and gas formation.

Behavior in Culture Media Containing Grape-Sugar.—Typhoid bacilli, dysentery bacilli, and *B. fæcalis alkaligenes* do not ferment grape-sugar, while most varieties of coli, and both types of paratyphoid bacilli do ferment it with the formation of gas (CO_2).

The test is made by stab-culture in 2 per cent. grape-sugar-agar, or by the inoculation of fermentation flasks containing 2 per cent. grape-sugar-bouillon.

Growth in Rothberger's Neutral Red Agar (cf. Chapter XII): Typhoid, dysentery bacilli and the bacillus fecalis

BIOLOGICAL CHARACTERISTICS OF TYPHOID
BE CONSIDERED IN

Bacteria	Motilitv	Behavior in—		
		Milk	Litmus-Whey	Sugar-Agar
Typhoid bacilli	Motile	No coagulation	Slight acid formation; clear	No fermentation
Bacterium coli	Non-motile or slightly motile	Coagulation	Liberal acid formation; clouded	Fermentation
Alkaligenes	Motile	No coagulation	Alkali formation	No fermentation
Dysentery bacilli	Non-motile	No coagulation	Slight acid formation; clear	No fermentation
Para-typhoid bacillus A	Motile	No coagulation	Slight acid formation; clear	Fermentation
Para-typhoid bacillus B	Motile	No coagulation	At first, acid formation; later, alkali formation	Fermentation

BACILLI AND OTHER BACTERIA WHICH MUST DIFFERENTIAL DIAGNOSIS .

Behavior in—				Indol Formation
Neutral-red-Agar	*Barsiekow's* Culture Medium			
	1 Per Cent. Grape-Sugar	1 Per Cent. Milk-Sugar	1 Per Cent. Grape-sugar; 1 Per Cent. Milk-sugar	
No reduction; no fermentation	Acid formation; coagulation	No acid formation; no coagulation	Acid formation; clouded	No indol formation
Reduction; fermentation	Acid formation; coagulation	Acid formation; coagulation	Acid formation; coagulation	Indol formation
No reduction; no fermentation	....	....	....	No indol formation
No reduction; no fermentation	Slight acid formation; no coagulation	Slight acid formation; no coagulation	Acid formation; clear	No indol formation
Reduction; fermentation	....	....	....	No indol formation
Reduction; fermentation	....	....	....	No indol formation

alkaligenes grow in this culture medium without changing it. Bacteria coli and paratyphoid bacilli discolor it after twenty-four hours' growth by reduction of the pigment and bring about a greenish fluorescence and gas formation owing to the sugar in the culture medium. The test is made by means of stab-cultures in well-filled test-tubes or still better with shake-cultures.

Growth in Loeffler's Green Solution (cf. Chapter XII).—After having grown from sixteen to twenty hours in *green Solution I*, the typhoid bacilli produce coagulation. Next to and above the smooth coagulum is a clear green fluid. Coli and paratyphoid bacilli also precipitate the nutrose, but with lively gas formation owing to the simultaneous fermentation of milk and grape-sugar; it does not form a smooth coagulum, but it looks torn and adheres to the wall of the test-tubes like a dirty green coating. On the surface a green foam-ring appears.

Green Solution II is not changed by the typhoid bacilli, but the coli bacteria ferment it, and the changes are the same as in green Solution I. The paratyphoid bacilli gradually discolor themselves from light-green into a pale-yellow without coagulating. With the aid of these two solutions, the coli bacteria, the typhoid and paratyphoid bacilli are differentiated from each other.

Indol Reaction.—Typhoid bacilli, in contradistinction to most varieties of coli bacilli, produce no indol, either when grown in bouillon or peptone water. *B. fæcalis alkaligenes*, dysentery, and paratyphoid bacilli also produce no indol.

Detection of Indol.—To 10 cc of a forty-eight-hour bouillon or peptone-water culture 1 cc of a 0.02 per cent. potassium nitrite solution and a few drops of chemically pure concentrated sulphuric acid are added. When indol is present a red coloration appears. On shaking with amyl alcohol the coloring matter is extracted, and can be more clearly seen. It is well always, as a control, to place in the incubator tubes which are inoculated with an authentic typhoid culture as well as uninoculated tubes.

Order of Examination of the Faeces for Typhoid Bacilli

I. ***Planting of Cultures from the Faeces.***—Thin stools of pasty or fluid consistency are used directly for planting cultures, while formed stools are first thoroughly mixed with a small quantity of sterile physiological salt solution.

1. On agar and the *Conradi-Drigalski* culture medium surface colonies are planted; for this purpose a right-angled glass spatula, which can be disinfected by burning with alcohol, or the ordinary platinum wire, is used. This is dipped into the material to be examined, and rubbed over the surface of the plate in all directions, and then smeared in the same manner upon a second, third, and fourth plate without being again sterilized or applied to the fæces. In this manner isolated surface colonies are obtained upon plates three and four. The *Conradi-Drigalski* plates should remain open for some time after the inoculation, until they have become absolutely dry, in order to guard against the coalescence of the developing colonies. The plates are then placed upside down—that is, with the cover down—in an incubator at 37° C.

2. *Inoculation of Urine-Gelatine.*—After this has been liquefied "dilution plates" are made in the usual manner —i.e., the first tube is inoculated with two loops of fæces. With four loops from this a second tube is inoculated, and with six to eight loops from the second tube a third is inoculated. The inoculated gelatine is then poured into plates, which after the medium has solidified on ice, are placed in an incubator at 21.5° to 22° C.

It is always well to inoculate several series of agar and *Conradi-Drigalski* plates.

II. ***Examination of the Plates.***—On the day after the inoculation the plates are tested in the following manner:

Agar and Conradi-Drigalski Plates.—From the agar plates only the small, transparent, bluish iridescent colonies need be considered for further examination; from the *Conradi-Drigalski* plates only the small, blue, sharply outlined colonies resembling dew-drops. Very minute portions are removed from these with a platinum needle, and examined in hanging-drops. When motile rods, having the appearance of typhoid bacilli, are seen, the remainder of the colony is transplanted upon slanting agar to obtain a pure culture. The so-called "preliminary agglutination test" may also be made at this time.

It is always necessary to remove a large number of suspicious looking colonies from the plates and to culture them for pure culture media.

Cultures made according to *Lentz* and *Tietz* are examined as follows: The blue plates are examined first for typhoid bacilli after they have been in the incubator sixteen to twenty hours. If no suspicious colonies are found, about 8 to 10 cc of 0.85 per cent. NaCl-solution are poured over the green plate which has remained in the incubator for twenty-four hours, and is then left standing quite still for two minutes. The fluid is now moved about on the plate a few times, whereby the loose typhoid and paratyphoid colonies are separated and left floating in the fluid, while the thick clusters of coli-colonies are segregated *in toto* and soon sink to the bottom. In order to produce the latter effect the plate is tilted, so that the fluid runs up to the edge; the thick clusters fall to the bottom after about half a minute, one to three loops are removed from the supernatent fluid and planted upon a *Conradi-Drigalski* plate (each loop is 2 mg) and rubbed up with a glass spatula on this and another blue plate according to the thickness of the colonies. If paratyphoid is suspected, malachite-green plates are used, in order to easier recognize the colonies of paratyphoid B. These plates are examined after standing in the incubator sixteen to twenty hours.

The pure cultures thus obtained are inoculated the day after for the purpose of determining their biological qualities in *Loeffler's* green solutions, litmus-whey, neutral red-agar; furthermore the quantitative macroscopic agglutination-test is made with a high-potency animal-immune-serum (cf. p. 272).

We recognize as typhoid bacilli such bacteria which correspond with these in their biological qualities and are agglutinated from a diluted solution of a high potency immune-serum which is of about the same titre as the serum.

We must, however, take note of the fact, that freshly inoculated typhoid bacilli very often do not agglutinate at all or with great difficulty, and that only after several inoculations on agar may agglutinate easier (cf. p. 272).

Paratyphoid bacilli are found in diseases which resemble typhoid or acute gastro-enteritis.

We distinguish two types, paratyphoid bacillus A and B, of which the latter is found very frequently, but the first extremely rarely.

Both types of bacteria look under the microscope like typhoid bacilli, but they differ from them and from each other by their behavior in culture-media, by their reaction to immunization, and by their animal pathogenesis.

The culture qualities of the paratyphoid bacilli have already been discussed when speaking of the differential diagnosis of the typhoid bacilli (cf. also table on pp. 110 and 111).

The paratyphoid bacilli A and B are distinguished positively from each other and from the other groups of typhoid coli by the agglutination-test with a high potency immune-serum.

Typhoid bacilli quite occasionally are influenced up to a certain degree by typhoid sera and typhoid bacilli by paratyphoid sera, this is, however, observed only in relatively concentrated solutions of sera and depends upon the presence of common partial agglutination.

In contradistinction to the typhoid bacilli the paratyphoid bacillus B is highly pathogenic for some animals, especially guinea-pigs and mice, and even very small doses are sufficient to deadly infect these animals $\frac{1}{10000}$ to $\frac{1}{1000}$ loop for an intraperitoneal injection, $\frac{1}{10}$ to $\frac{1}{20}$ loop for a subcutaneous inoculation. The animals die of septicemia. The paratyphoid bacillus A kills mice when mixed with the food.

The paratyphoid bacilli are demonstrated in the fæces the same way as the typhoid bacilli.

As culture media are used likewise litmus-lactose, fuchsine-agar and malachite-green agar.

The paratyphoid bacillus A grows in these media the same way as the typhoid bacilli. Paratyphoid bacillus B forms blue colonies on *Conradi-Drigalski* plates, which mostly, but not as a rule, are larger, juicier, and less transparent than typhoid-colonies.

The colonies of paratyphoid bacillus B appear colorless on fuchsine-agar, the same as the colonies of the typhoid bacilli, but they are occasionally larger and fuller developed.

Paratyphoid bacillus B forms in malachite-green agar after having grown in the incubator sixteen to twenty hours, transparent, slightly milky colonies of 2 to 3 millimetres diameter which stain the zone around them yellow. The malachite-green agar offers especially to the paratyphoid bacillus B very favorable conditions to grow, and is, therefore, very serviceable for cultures from the fæces, especially if they have gone through the preliminary culture according to *Lentz* and *Tietz.*

The paratyphoid bacilli are identified by their cultural qualities, by their pathogenity in animals, and by the agglutination test with a high potency immune serum.

Bacilli in Meat-Poisoning (Enteritis Bacilli)

The bacteria found in meat poisonings under gastro-intestinal symptoms may be divided into two groups.

The first group, type *Aertryck* (according to *De Nobili*), has the same cultural qualities as paratyphoid bacillus B and cannot be separated from it by the reactions for immunization. They may be bacilli, which are identical with the paratyphoid bacillus B. The bacilli of the so-called hog-cholera group (B. typhi murium *Loeffler*, bacillus of the hog-pest, psittacose-bacillus) also belong to this group.

The second group, type *Gaertner* (according to *De Nobili*), is also culturally identical with the paratyphoid bacillus B, but differs from it by its sero-diagnosis. In this group also belong the rat-pathogenic bacilli of *Danysz*, *Issatschenko*, and *Dunbar's* rattin. The detection of these bacteria is accomplished by the method given for typhoid and paratyphoid.

Dysentery Bacilli

Based upon numerous investigations carried out withih the last few years, it can be assumed that in all probability the bacillus, cultivated first by Shiga, and two years later by *Kruse*, from the bloody mucus evacuations of dysentery patients, is the exciting cause of the epidemic dysentery, which appears chiefly in countries of the North Temperate Zone.

This disease must not be confused with that caused by amœbæ, now usually designated as amœbic dysentery.

Characteristics of Dysentery Bacilli.—Dysentery bacilli are rods about the length of typhoid bacilli, but somewhat thicker and plumper. In contradistinction to the latter, they are non-motile. They stain easily with dilute aniline dyes, and are decolorized by *Gram.*

The dysentery bacilli grow in all the usual media. Their cultures develop a distinct odor after sperma. They form the same as the typhoid bacilli in agar round, flat cultures, which are whitish in ordinary light and moist, in transmitted light bluishly iridescent.

If grown in gelatine their superficial colonies after forty-eight hours are similar to those of the typhoid bacilli. They also show the so-called wine-leaf shape. They do not liquefy gelatine. If grown on potatoes which are of a weak acid reaction, or in bouillon, they do not differ from typhoid bacilli. In the culture media of *Conradi-Drigalski* they form round dewdrop-like colonies, which show a faint milky cloudiness, and they do not change the blue color of the culture media.

As to the biological characteristics of the dysentery bacilli cf. pp. 110 and 111.

They are distinguished from the typhoid bacillus because of their immotility and their behavior in *Barsiekow's* solution; from the paratyphoid bacillus because of their behavior in sugar and neutral red agar; from the coli bacilli because of their growth in milk, litmus-whey, sugar agar, neutral red agar, etc. (cf. table, pp. 110 and 111).

For the positive identification of the dysentery bacillus is the agglutination with a specific serum of a high potency.

The *Shiga-Kruse* bacillus is to be distinguished from the *Flexner* and the pseudo-dysentery bacilli through its behavior in nutritive media, which contain mannit. The *Shiga-Kruse* bacillus does not decompose mannit; the *Flexner* and the pseudo-dysentery bacilli cause fermentation of the mannit with the production of acids without the production of gases. To test this behavior we use *Barsiekow's* nutritive solution with one per cent. mannit or the litmus mannit agar (cf. Chapter XII). The *Shiga-Kruse* bacillus does not change the color of the *Barsiekow* solution, the other bacteria color the solution red and coagulate the albumin. In litmus-mannit-agar stab-cultures may be made. The *Shiga-Kruse* bacillus reduces the litmus to a lighter color in the deeper layers; the *Flexner* and the pseudo-dysentery bacillus color the solution a reddish violet after twenty-four hours, and a distinct red after forty-eight hours. Instead of stab-cultures we can also get the cultures upon the surface of this nutritive medium which has been put in *Petri* dishes. The colonies of the *Shiga-Kruse* bacilli leave the color of the nutritive media unchanged, the *Flexner* and the pseudo-dysentery bacilli stain the media red after forty-eight hours.

Another means of differentiation is the agglutination test. In a serum produced by the *Shiga-Kruse* bacilli, the *Flexner* bacilli are agglutinated only in concentrated solutions, and vice versa. It is therefore necessary to have a thorough titration of the serum in order to be able to make a positive diagnosis.

In the animal experiments the *Shiga-Kruse* bacilli proved more poisonous than the *Flexner* and pseudo-dysentery bacilli. The intravenous injection of one-twentieth per cent. of the living culture, or of a 60 per cent. of the dead culture of the *Shiga-Kruse* bacilli kill rabbits in from one to three days, producing paralysis.

The dysentery bacilli are easily demonstrated so long as the fæces contain blood and mucus. Occasionally the examinations must be repeated several times. If the evacuations have again become of fecal consistency they are not so easily or, possibly not at all, demonstrable. The best nutritive medium is the *Conradi-Drigalski* agar, in which the sugar of milk was substituted by mannit of a like concentration.

Order of Examination.—A smear is made from a mucus fleck and stained with dilute carbol-fuchsin. Dysentery bacilli are frequently detected in it in almost pure culture, only a few coli bacilli, as a rule, being present with them. Other mucus flecks are used for the inoculation of cultures. Gelatine plates are made in the usual manner, and smears upon agar and the *Conradi-Drigalski* culture medium, as in the examination of the fæces for typhoid bacilli.

The gelatine plates are examined forty-eight hours after inoculation, the agar plates sixteen to twenty-four hours. Pure cultures are obtained from the suspicious-looking colonies, and are tested for their biological characteristics, and the agglutination test is carried out with a high-potency immune serum (cf. Examination of Typhoid Plates, p. 114).

Cholera (Plate V, Fig. H)

Characteristics of the Cholera Vibriones.—Cholera vibriones are very motile, slightly curved, short rods, which stain easily with dilute aniline dyes (carbol-fuchsin, 1 to 10), and are decolorized by *Gram.* In stained smears from pure cultures numerous bacilli lying close together in semicircular or S-shaped figures, or, especially in old cultures, in spirally interwoven threads, are frequently seen. Cholera vibriones grow easily upon all the usual culture media, especially in the presence of a marked alkaline reaction.

Upon agar they develop, after eighteen to twenty-four hours' growth at 37° C., small transparent colonies, which, when held against the light, have a bluish iridescence. They can be easily distinguished from colonies of most of the other bacteria present in cultures from the fæces by their transparency when examined in direct light.

On gelatine, after twenty-four hours' growth at 22° C., cholera colonies appear to the naked eye as very small, bright points. When examined with the low power, they appear as small, round, glistening discs with irregular wavy margins. The surface of the colonies is granular and highly refractive, so that they appear as though sprinkled with fine particles of glass. Gelatine is slowly liquefied.

In bouillon, cholera vibriones grow luxuriantly, clouding it evenly, and forming a film on its surface.

Milk is not coagulated; blood-serum is liquefied.

Alkaline peptone-water is an especially favorable culture medium for cholera vibriones, as, in fact, for all vibriones. When material which contains other microorganisms in addition to cholera bacteria is placed in peptone-water, a marked increase of the vibriones takes place, especially in the upper portion of the solution, where they grow much faster and more luxuriantly than the other bacteria present. Frequently, even after but six hours' growth at 37° C., very motile aerobic vibriones are present in pure culture on the surface of the culture media.

If a few drops of concentrated, chemically pure sulphuric acid are added to a twenty-four hours' peptone-water culture of cholera vibriones, a violet coloration appears, which may be extracted by shaking with amyl alcohol (cholera-red reaction). This color reaction depends upon the fact that the bacteria when grown in peptone-water produce a large amount of indol, and reduce the nitrates present in the media to nitrites. Upon the addition of sulphuric acid, nitrous acid is formed, which unites with the indol and produces the red color (nitroso-indol reaction). This reaction is by no means, as was formerly thought, a peculiarity of cholera vibriones, but is produced by a number of other vibriones. It has, however, a value in differential diagnosis, as it is a constant characteristic of cholera vibriones, and its absence, therefore, is proof that the bacteria examined are not cholera vibriones. Of course, a control must always be made to see if an authentic cholera culture gives the red reaction when grown in a tube of the same peptone-water.

Serum diagnosis affords the most valuable means for differentiating between cholera vibriones and other closely related varieties of vibriones which may be present in the fæces with them. Only such vibriones as are agglutinated by a high dilution of a serum obtained from an animal immunized with cholera vibriones, and are dissolved by the bacteriolytic elements of such a serum, in the manner prescribed in *Pfeiffer's* test, should be considered as true cholera vibriones.[1]

The agglutination test is carried out in the manner described under Examination of the Fæces for Typhoid Bacilli.

Pfeiffer's test depends upon the fact that when an animal is immunized with cholera vibriones, in addition to agglutinin, bacteriolytic substances appear in the serum. If such immune serum is injected together with cholera vibriones into the peritoneal cavity of a guinea-pig, and after twenty minutes to one hour a few drops of the peritoneal contents are withdrawn with a capillary-tube and examined in a hanging-drop, vibriones are no longer found, but in their place small, pale spherules. Finally, these disappear and the cholera vibriones are completely dissolved by the bacteriolytic substances contained in the serum. This reaction is absolutely reliable, as the bacteriolytic action of cholera immune serum is directed only against cholera vibriones, never against other bacteria.

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[1] Serum used for the agglutination test and for *Pfeiffer's* test can be obtained from the Institut für Infektionskrankheiten in Berlin.

Method of Carrying Out Pfeiffer's Test

The serum used for this test should have as high a potency as possible; this should be at least so high that 0.0002 gramme is sufficient to dissolve, within half an hour with the formation of spherules, the cholera bacteria contained in a mixture of one loop (1 loop = 2 milligrammes) of an eighteen hours' agar cholera culture of standard virulence, with 1 cc of nutrient bouillon, when injected into the peritoneal cavity of a guinea-pig—i.e., the serum must have a titre (standard of potency) of at least 0.0002 gramme.

For *Pfeiffer's* test, four guinea-pigs, weighing 200 grammes each, are necessary.

Animal A receives five times the titre dose—i.e., 1 milligramme of a serum whose titre is 0.0002 gramme.

Animal B receives ten times the titre dose—i.e., 2 milligrammes of the serum.

Animal C serves as control-animal and receives fifty times the titre dose—i.e., 10 milligrammes of normal serum from the same kind of animal as that from which the serum used with animal A and B was obtained.

Each animal receives the serum mixed with one loop of the culture to be examined, grown on agar for eighteen hours at 37° C., in 1 cc of bouillon (not in salt solution or peptone solution), injected into the peritoneal cavity.

Animal D receives a quarter of a loop of the cholera culture intraperitoneally, in order to ascertain if the culture is virulent for guinea-pigs. A blunt cannula is used for the injection. The injection is made into the peritoneal cavity through a cut in the skin; the cannula can be easily forced into the peritoneal cavity. The peritoneal exudate is withdrawn for microscopical examination, at the same point, by means of a capillary glass-tube.

The exudate is examined in a hanging-drop, with the high power, twenty minutes after, and one hour after, the injection.

In animal A and animal B, after twenty minutes or, at the latest, after one hour, the typical spherules must have formed or the vibriones must have dissolved; while in animals C and D a great quantity of highly motile, well-preserved vibriones must be present. For the identification of convalescent cases of cholera, *Pfeiffer's* reaction must be carried out in the following manner: Dilutions of serum from the suspected person, in proportions of 1:20, 1:100, 1:500, are made with bouillon. From these 1 cc is taken, mixed with one loop of an eighteen-hour agar culture of virulent cholera vibriones, and injected into the peritoneal cavity of guinea-pigs weighing 200 grammes each. A control-animal receives an intraperitoneal injection of a quarter of a loop of the same culture, dissolved in 1 cc of bouillon, but without serum. A positive result of the reaction after twenty to sixty minutes indicates that the person from whom the serum was taken has had cholera. (Instructions of the Prussian Ministry entrusted with the Control of Religious, Educational, and Medical Affairs, in Regard to the Bacteriological Diagnosis of Cholera, November 6, 1902.)

Detection of Cholera Vibriones in the Faeces

Detailed instructions concerning the examination of the fæces for cholera bacteria are contained in the above-mentioned order.

1. ***Microscopical Examination.***—Smears are made, when possible, from a mucus fleck, and stained with dilute carbol fuchsin (1:9). Frequently, typical comma bacilli are present in these smears in great numbers, or even in pure culture, arranged in characteristic shoals. In many cases, however, the bacteria are not present in such large numbers, and cannot be recognized among the great number of bacteria normally present in the intestines.

In addition, hanging-drops are made from a sputum fleck with peptone solution, and examined fresh and stained, at once and after half an hour, in an incubator at 37° C. Occasionally the vibriones are seen to collect at the margin of the drop.

A certain diagnosis can never be made from the microscopical examination alone, but cultural procedures must always be used.

2. ***Gelatine Plates.***—Two tubes of melted gelatine are inoculated with one loop of the material to be examined (when possible, from a mucus fleck) and two dilutions made in the usual manner by transplanting three loops at a time. The gelatine is poured into plates and examined after eighteen hours' growth at 22° C.

3. ***Agar Plates.***—Agar plates must be absolutely dry. They are, therefore, placed, open and with the surface down, in an incubator for half an hour before they are inoculated. A loop from the fæces or a mucus fleck is planted upon a series of plates, in the manner described under the examination for typhoid bacilli. The plates are examined after twelve to eighteen hours' growth at 37° C.

4. ***Enrichment by Means of Peptone Solution.***—Six tubes, containing 10 cc each, are inoculated; each tube receiving one loop of fæces. When it is suspected that very few cholera vibriones are present in the stools, 1 cc of fæces is covered in a flask with 50 cc of peptone solution. After six to twelve hours' growth in an incubator at 37° C., a small portion is withdrawn from the surface of the peptone solution without disturbing the rest, and examined microscopically. Frequently, a pure culture of vibriones is seen in the specimen. Gelatine and agar plates are inoculated from the tube containing the most suspicious-looking bacteria. If pure cultures do not grow on these plates, the suspicious-looking colonies are removed and transplanted on agar tubes.

Finally, serum tests are carried out with the pure cultures (quantitative macroscopical agglutination test and *Pfeiffer's* reaction). It must be remembered that vibriones resembling cholera vibriones, which may be present in the fæces, are also increased in peptone-water, and that they cannot be distinguished upon agar and gelatine from true cholera vibriones.

The diagnosis of cholera is considered certain when all these tests are positive.

The first cases of an epidemic should always be examined in this thorough manner. Later in the epidemic, cultural examination and the preliminary agglutination test in a hanging-drop (cf. p. 272) suffice, when the latter gives a definite result.

Tubercle Bacilli

The detection of tubercle bacilli in the fæces is accomplished by means of stained smears. It succeeds most easily in smears made from the flecks of mucus and pus, which are present in the diarrhœal evacuations of patients suffering from intestinal tuberculosis.

If the fæces to be examined are formed, they are (according to *Strassburger*) mixed with water and centrifugalized. The cloudy liquid above the sediment is poured off and diluted with 96 per cent. alcohol (two parts fluid to be examined, one part alcohol). It is then centrifugalized again and smears made from the sediment.

Care should be exercised in forming an opinion from the smears. Aside from the fact that a negative result is, of course, in no case proof against tuberculosis, it should always be borne in mind when the result is positive that the tubercle bacilli may have gained entrance to the intestines in sputum which has been swallowed. Moreover, acid-fast bacilli which were not tubercle bacilli, have been repeatedly found in the fæces.

The case is in all probability one of intestinal tuberculosis, when in repeated examinations numerous acid-fast bacilli, having the appearance of tubercle bacilli, are found in the fæces of patients who have certainly swallowed no sputum.

Staphylococci and Streptococci

These bacteria appear in the fæces both as the exciting cause of acute intestinal catarrh, and following the rupture of abscesses into the intestines. In the first case, the pyogenic bacteria are seen in such great numbers that the micro-organisms normally present in the stools are completely overshadowed by them. Their detection is accomplished by means of smears stained with dilute carbol-fuchsin, and according to *Gram.*

Anthrax Bacilli

In the rare cases of intestinal anthrax, anthrax bacilli are evacuated in the fæces. Their detection is accomplished by means of cultures. Cultures are planted upon gelatine and agar, the characteristic colonies (cf. p. 300) are removed, grown in pure culture, and for their identification injected into white mice.

Plague Bacilli

Plague bacilli have been found in a few cases in the fæces of plague patients. Their detection is accomplished by means of animal inoculation, the fæces being rubbed into the shaved abdomen of guinea-pigs

CHAPTER VII

EXAMINATION OF THE URINE

I. Collection of the Urine

The twenty-four hours' quantity is usually collected in a receptacle which has been thoroughly cleansed with hot water. To guard against the decomposition of the urine, it is well to add a piece of thymol the size of a bean, or 10 to 20 drops of chloroform. If, in spite of this, the urine decomposes quickly, and shows a pronounced alkaline reaction, it is well to request a freshly passed sample of urine for examination, in addition to the twenty-four hours' sample. In many cases it is necessary to examine different portions of the daily output separately. In affections of the kidney the morning and evening urine are separately examined; in mild cases of diabetes or in alimentary glycosuria, that passed before and after meals. When there is suspicion of a physiological or cyclic albuminuria, every sample of urine passed must be separately examined for albumin. For purposes of differential diagnosis in diseases of the urethra and bladder, a single sample (preferably the first morning urine) is collected in two or three vessels, and each portion examined separately for its physical, chemical, and microscopical characteristics. Catheterization of the ureters, which has been recently introduced into practice, renders it possible to isolate the urine of each kidney, and by this means to determine with certainty, in cases of unilateral disease, which of the two kidneys is affected. For this purpose the so-called segregators, by means of which the bladder is divided into two parts by a partition, are also used. This latter method is, however, not entirely free from objections, and in cases in which catheterization of the ureters is not contraindicated, it is to be preferred to the use of the segregator. In collecting urine care must always be taken that no foreign matter (sputum, menstrual blood, spermatic fluid, etc.) becomes accidentally mixed with it.

Concerning the Collection of Urine for Bacteriological Examination, cf. p. 237.

II. The Identification of a Fluid as Urine

In practice, it is occasionally necessary to be able to identify with certainty as urine a fluid which has been submitted for examination. This may be necessary in many cases, owing to the suspicion of accidental or intentional substitution on the part of the patient. In other cases the identification of a fluid as urine is absolutely indispensable for purposes of differential diagnosis. This latter is especially true of fluids aspirated from the region of the kidneys. In such cases the differential diagnosis lies usually between hydronephrosis and cystic tumors, or echinococcus.

To identify a fluid as urine, some of the constituents characteristic of urine alone must be detected in it. Of the numerous organic and inorganic constituents of the urine, urea and uric acid are considered characteristic, and their simultaneous presence in a fluid is recognized as sufficient evidence that the fluid is urine. If, in addition, a third constituent of the urine—creatinin—is detected, the fluid examined may be considered with certainty as urine. These constituents of the urine are detected in the following manner:

Urea: $CO(NH_2)_2$.—Twenty-five to 50 cc of urine are evaporated in a porcelain dish to a thin syrup. After this has cooled, a few cc of concentrated nitric acid are added. This causes a precipitation of crystals of urea nitrate. Microscopical examination reveals typical rhomboid plates lying upon one another.

If there is but little fluid, a few drops of it are placed on a slide, a drop of nitric acid added, and the slide warmed carefully over a small flame. On cooling, crystals of urea nitrate are precipitated.

Pure urea may be obtained from the urea nitrate in the following manner:

The crystalline precipitate is collected on a filter, dried between two pieces of filter-paper, and dissolved in a small quantity of water. The urea nitrate is then decomposed with barium carbonate, the urea extracted from the dried mass with absolute alcohol, and the liquid decolorized with animal charcoal. When the colorless alcoholic solution, which has been concentrated by heating, cools, urea crystallizes in the form of needles. The biuret reaction is characteristic of pure urea. A few crystals are carefully heated in a dry test-tube until dissolved. By this process ammonia and biuret ($C_2H_5N_3O_2$), which, when dissolved in water, gives the typical biuret reaction, are formed. (For the method of carrying out the biuret reaction, cf. p. 149.)

Uric Acid: $C_5H_4N_4O_3$.—Fifty to 100 cc of urine are rendered strongly acid by the addition of hydrochloric acid. After some hours' (twelve to twenty-four) standing, uric acid is precipitated in the form of yellowish-brown crystals. These crystals are collected upon a small filter, washed several times with water, a portion placed in a porcelain dish, and the murexide test carried out; 2 to 3 drops of concentrated nitric acid are added, and the mixture carefully heated over a flame until the nitric acid is driven off. The dry residue becomes violet upon the addition of a drop of sodium or potassium hydrate, purple-red upon the addition of ammonia.

Creatinin: $C_4H_7N_3O$.—Ten to 15 cc of urine are treated with a few drops of a dilute solution of sodium nitroprusside, and dilute sodium hydrate is added drop by drop. If creatinin is present, the fluid assumes a beautiful ruby-red color, which remains but a short time (in dilute solutions only a few minutes). Gradually the red color passes into a straw-yellow. If this yellow fluid is treated with glacial acetic acid (5 to 10 drops) and heated, it becomes at first green, then blue; on longer standing, a blue precipitate is thrown down.

III. Chemical and Physical Characteristics of the Urine

1. ***Color.***—Normal urine may have any shade of color between pale yellow and reddish-brown. Ordinarily, the color of the urine is proportional to its concentration. Upon standing in the air, the normal acid urine usually becomes darker, which is probably due to the conversion of the chromogens into pigments, as the result of oxidation. Abnormal color of the urine may be due either to pigments formed in the body, drugs, or articles of food. Among the abnormal pigments of the urine are:

(*a*) *Blood-Pigment.*—The urine is colored from pinkish red to brownish-black.

(*b*) *Biliary Pigments.*—The urine is colored yellowish-green to dark brown.

(*c*) *Melanin* causes a dark brown to black color. The freshly passed urine contains only melanogen, and is not deeply colored. The pigment forms gradually upon standing in the air, or upon the addition of oxidizing substances.

Drugs and articles of food may cause the following variations in the color of the urine:

A brownish-yellow to brownish-black coloration following the internal or external use of carbolic acid, salicylic acid, preparations of cresol, brenzkatechin, tar, folia uvæ ursi, and similar preparations, which are excreted in the urine in combination with sulphuric acid.

Urine has a golden or lemon-yellow color when its reaction is acid, and a bright red color when it is alkaline, after the internal use of rheum, senna, cascara sagrada, chrysarobin, and similar preparations containing chrysophanic acid. Following the use of santonin, the urine is, if acid, greenish or saffron-yellow in color; if alkaline, red.

Antipyrin, sulphonal, and trional cause a yellow to blood-red color.

Following the internal use of methylene blue, the urine is colored blue or green.

2. ***Transparency.***—The various constituents of normal urine are in solution; freshly passed normal urine is, therefore, perfectly clear and transparent. However, a very small quantity of swollen albuminoid and mucoid substances, which come from the surface of the bladder and the urethral mucosa, are present, and after standing a short time, form small clouds, nubecula, and sink gradually to the bottom of the vessel.

If the urine is cloudy when passed, or if it becomes cloudy soon after being passed, abnormal or pathological conditions may be present; at any rate, the cause of the cloudiness must be determined in every individual case, as it is of the greatest importance for diagnosis and treatment.

Cloudy urine may be due to the following causes:

1. Urinary salts suspended in the urine.

2. The presence of many cellular elements from the urinary tract (blood, pus-corpuscles, epithelium).

3. Bacteria.

4. A milky cloudiness may be produced by fat in emulsion (chyluria and lipuria).

In order to determine the cause of the cloudiness it is well to proceed systematically as follows:

(*a*) A sample of the urine in a test-tube is first carefully warmed over a flame; if the urine clears, the clouding was due to urates. These are often precipitated when the urine has been allowed to stand, and form the well-known brick-dust sediment (*sedimentum lateritium*). If this uratic cloudiness is accompanied by that due to other substances (usually cellular elements), the urine does not become entirely clear when heated, but merely clears a little, and may even, if the heating is continued, become clouded again (by precipitation of albumin).

(*b*) If heating causes no change in the cloudiness, 10 to 15 drops of acetic acid are added; if this renders the urine entirely or partially clear, the cloudiness was due principally to phosphates. The urine often does not become perfectly clear, since in such cases the reaction is, as a rule, alkaline, and the urine, being somewhat decomposed, usually contains, in addition, numerous bacteria or cellular elements. If acetic acid also exerts no influence, then:

(*c*) Hydrochloric acid is added. If the cloudiness now disappears, it was due to calcium oxalate.

(*d*) If the cloudiness is uninfluenced by these three procedures, the urine is first treated with sodium hydrate (10 per cent.) and shaken. If in place of the cloudiness a gelatinous transparency appears, the cloudiness was due to pus (*Donné's* test for pus). This test depends upon the property of pus-corpuscles to swell under the influence of alkalies and form a cohesive jellylike mass.

(*e*) If the cloudiness is due to fat, the urine becomes entirely clear upon the addition of alcohol and ether. If the cloudiness resists all these procedures, it is, in all probability, due to bacteria. In such cases the urine is evenly cloudy; it forms no noticeable sediment upon standing, and usually remains turbid, even after repeated filtration. If such urine is held in a test-tube against the light and lightly shaken, the cloudiness is seen to have a fluorescent, wavy character.

3. ***Reaction.***—The reaction of the urine may be acid, amphoteric, or alkaline. Normal urine is usually acid in reaction. Its acidity is not due to the presence of free acids, but to salts with acid reaction, principally to acid sodium phosphate (mono-sodium phosphate). In addition to the acid phosphates, alkaline phosphates are also present in the urine. Their quantity is usually small in comparison with the acid phosphates, which explains the acid reaction.

If the alkaline phosphates are increased, an amphoteric or alkaline reaction is produced. The reaction is called amphoteric when the urine reacts both alkaline and acid, which is due to the fact that the phosphates with acid reaction and those with alkaline reaction are present in the urine in such proportions that they exert an equal influence upon its reaction. The reaction of the urine is alkaline, when a large proportion of basic phosphates are present.

The urine may be alkaline, in pathological conditions, or due to decomposition after standing in unclean receptacles. Alkaline or ammoniacal fermentation of the urine is the conversion of urea into ammonium carbonate, caused by micro-organisms (*Micrococcus ureæ*, *Proteus vulgaris Hauseri*, and others). The urine becomes unpleasant and ammoniacal in odor and cloudy, due to the precipitation of alkaline and earthy phosphates, ammonium magnesium phosphate, ammonium urate, and calcium carbonate.

The reaction of the urine is determined in the usual manner with litmus-paper. When the reaction is acid, blue litmus-paper is turned red; when alkaline, red is turned blue; and when amphoteric, both reactions are equally pronounced. In ammoniacal decomposition of the urine the blue color of the litmus-paper disappears on drying in the air. In addition, ammoniacal urine is characterized by the fact that, when a glass rod wet with hydrochloric acid is held over it, white clouds are formed (sal-ammoniac).

4. **Specific Gravity.**—The specific gravity of the urine varies under physiological conditions between 1.005 and 1.030, and depends principally upon the amount of water ingested, and the amount excreted by other organs.

The simplest method of determining the specific gravity is by means of two urinometers, with divisions from 1.000 to 1.025 and 1.025 to 1.050. A rather wide glass cylinder is filled about four-fifths full of the urine to be examined, and the dried urinometer carefully placed in it.[1] The degree upon the scale to which the urinometer sinks is noted. If the urinometer sinks too deeply (beyond the scale), or does not sink to the scale, the specific gravity must be determined with the other urinometer. For exact determination the temperature must be taken into account. Urinometers are usually corrected for 15° C. If the temperature of the urine is higher, one degree (0.001) must be added for each three degrees of the thermometer. If the temperature is under 15° C., the same amount must be subtracted.

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[1] The bubbles of foam which frequently form must be removed with filter-paper.

From the specific gravity of the urine the total solids dissolved in it can be approximately calculated. For this purpose, according to *Haeser*, the last two places of the specific gravity, which is determined to three decimals, are multiplied by 0.233. If, for example, the specific gravity of the urine is 1.025, the percentage of solids present is $25 \times 0.233 = 5.825$ per cent.

5. **The Freezing-Point of the Urine.**—As early as the eighteenth century (1788) *Blagden* had discovered that a simple relation exists between the temperature at which salt solutions freeze and the amount of dissolved matter in the solutions—namely, that the two are proportional. This work was, however, entirely forgotten. It was only after *Raoult* and *van't Hoff* had found simple laws for this fact that it was turned to scientific use. These laws are:

1. Equimolecular solutions—i.e., solutions whose concentrations are proportional to the molecular weights of the substances dissolved have the same freezing-point.

Example.—The molecular weight of grape-sugar ($C_6H_{12}O_6$) is 180, that of urea (CON_2H_4) 60. A solution which contains 180 grammes of grape-sugar to the litre, and a solution which contains 60 grammes of urea to the litre, have the same freezing-point. From this it follows that the freezing-point does not depend (as does the specific gravity) upon the mass of the substances dissolved, but upon the number of molecules in solution, and therefore can be considered as a measure of the molecular concentration of the solution.

2. The freezing-point of a solution is proportional to its osmotic pressure.

The determination of the freezing-point of urine and of blood was first introduced into clinical practice by *v. Koranyi*, and within a short time has come into comparatively widespread use, especially in the diagnosis of functional kidney disease.

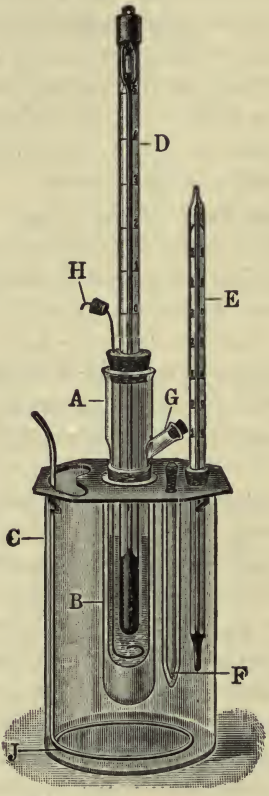

FIG. 22

For practical purposes the freezing-point of the urine is best determined by means of *Beckmann's* apparatus (kryoskop).[1] The apparatus is composed of the following parts: Glass A (Fig. 22) contains a thermometer D, which is divided into 0.01 degree, and a stirrer H of bent platinum wire. This glass is set in another somewhat wider glass B, which serves as an air-jacket. The latter is fastened in the cover of a strong large jar C. In the cover of this large jar are also: (1) An ordinary thermometer for determining the temperature of the freezing-mixture; (2) a tube which contains a glass rod F. By means of the strong stirrer I the temperature of the freezing-mixture is kept even. The freezing-point of the urine is determined in the following manner: Jar C is filled about three-quarters full of a mixture of water, ice, and common salt. The temperature of the freezing-mixture must not be lower than 5° to 6° C. The urine to be tested is placed in Glass A; its quantity should be just sufficient to completely cover the mercury reservoir of thermometer D. In order to obtain even cooling of the urine, the freezing-mixture and the urine are constantly stirred by means of stirrers H and I. The moment in which the urine freezes is indicated by the fact that the mercury column of the thermometer, which has been up to this time sinking, suddenly rises and remains at a definite point. The rising of the mercury column is due to the fact that before freezing the fluid is always slightly overcooled, and the thermometer therefore falls below the freezing-point. As soon as the fluid freezes, it is warmed to the temperature of the freezing-point, and therefore the mercury column rises to this point. It frequently happens that, in spite of marked overcooling, the fluid does not freeze. In such a case a small particle of ice is introduced into the fluid through tube G, by means of rod F. The urine freezes at the instant in which the ice comes in contact with it. Since *Beckmann's* thermometer has no constant zero-point, the latter must be established by determining the freezing-point of distilled water, and must be frequently controlled; this also applies to those forms of apparatus which have a constant zero-point, since, as experience has shown, the zero-point in such forms of apparatus varies after a time, and no longer agrees with that marked upon the scale.

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[1] Recently numerous simplified forms of apparatus for clinical use have been suggested. The simplifications have, however, unfortunately always been made at the expense of accuracy.

6. ***Quantity.***—The daily quantity (twenty-four-hour) of the urine is very variable, even under physiological conditions. The ingestion of water, the excretion of water by means of the skin and lungs, the ingestion of diuretic substances (alcohol, tea, coffee), muscle activity, psychic and other agencies, influence greatly the excretion of urine, and therefore the quantity of urine passed in twenty-four hours. On the average, the quantity passed by a healthy man is 1,500 cc, by a woman somewhat less.

Normally, the quantity of the urine is in inverse proportion to its specific gravity. For estimating the daily quantity, the urine is usually collected in a large glass vessel, well shaken, and its volume measured with a graduated cylinder. To prevent decomposition of the urine by bacteria, it is well to add to it a piece of thymol the size of a bean. The weight of the urine can be easily calculated from its measured volume by multiplying the latter by the specific gravity. For example, 1,500 cc of urine of a specific gravity of 1.025 would weigh $1,500 \times 1.25 =$ 1537.5 grammes.

IV. The Chemical Examination of the Pathological and Abnormal Constituents of the Urine

1. Albuminoid Bodies in the Urine

Normal urine is free from albumin. The very small amount which can be obtained from a large quantity of urine does not come into consideration in the clinical examination, since it cannot be detected by the usual tests for albumin. In abnormal and pathological conditions, the urine may contain the following varieties of albumin:

(*a*) Serum-albumin.
(*b*) Serum-globulin.
(*c*) Albumoses and peptones.
(*d*) Fibrin.
(*e*) Nucleo-albumin.

These albuminoid substances are distinguished by the following general and physical characteristics:

SERUM-ALBUMIN is soluble in water, dilute salt solutions, and saturated solutions of sodium chloride and magnesium sulphate. It is precipitated by ammonium sulphate; its coagulating temperature is 72° to 75° C. It is precipitated and converted into acid-albumin by concentrated mineral acids, but not by dilute; it is converted by alkalies into alkali albumin. Acid-albumin is soluble in acetic acid.

SERUM-GLOBULIN.—Insoluble in water, soluble in dilute salt solutions, and insoluble in concentrated solutions of sodium chloride, magnesium sulphate, and ammonium sulphate. It is coagulated by heat (75° C.).

ALBUMOSES are intermediate products in the hydrolysis of proteids (the end-products are peptones). They correspond to propeptone, and partially to peptone, as understood by *Brueckes*. They are not coagulated by heat.

They give a rose-red coloration with the biuret reaction. Three varieties of albumoses are differentiated (according to their solubility).

(*a*) *Protalbumoses.*—Soluble in hot and cold water and salt solutions. They are precipitated by saturation with sodium chloride and magnesium sulphate.

(*b*) *Hetero-albumoses.*—Insoluble in water; soluble in 0.5 to 15 per cent. sodium chloride solution at ordinary temperatures. On heating to 65° C. they are precipitated. The precipitate is soluble in dilute alkalies or acids. Proto- and hetero-albumoses are frequently designated as primary albumoses.

(*c*) *Deutero-albumoses* (*Secondary Albumoses*).—Soluble in cold and hot water; are not precipitated by saturation with sodium chloride or magnesium sulphate, but are precipitated by saturation with ammonium sulphate.

FIBRIN is insoluble in water as well as in dilute acids and alkalies. By the latter it is converted at ordinary temperatures to a jelly, which dissolves after long boiling.

NUCLEO-ALBUMIN is insoluble in acetic acid, and is precipitated from solutions by it. Nucleo albumin contains phosphorus.

2. Detection of Albumin in the Urine

Two varieties of albuminuria are distinguished: an accidental or false albuminuria (albuminuria spuria), and a true or renal albuminuria (albuminuria vera). The first is due to the admixture of fluids which contain albumin, such as blood, pus, and chyle, with urine, which, when secreted, was free from albumin. Renal albuminuria is caused by parenchymatous changes, or disturbances of circulation in the kidney.

In both varieties of albuminuria, the albumin in the urine is composed of a mixture of serum-albumin and serum-globulin. Since these two albuminoid bodies give the same reactions, and their division is unnecessary for clinical purposes, albumin and globulin will be estimated as one in the following tests for albumin.

The examination of the urine for albumin must be carried out with the greatest care, as even the slightest possible detectable quantity of albumin has a diagnostic significance.

Urine which is to be examined for albumin must be:

1. Absolutely clear.
2. Acid in reaction.
3. Free from contamination with albuminoid or other secretions (menstrual blood, sputum) which do not come from the urinary tract

The salts, which are suspended in the urine, bacteria, and cellular elements are removed by filtration. If the urine is not clear after filtration, it is treated with magnesium carbonate, barium carbonate, or marble-dust, well shaken, and again filtered. The substances producing the cloudiness are collected by the precipitate, and a clear filtrate is obtained. Urine in which the cloudiness is due to precipitated urates can be most easily clarified by slight heating.

Of the numerous reactions recommended for the detection of albumin in the urine, only those will be mentioned here which have been found in practice to be reliable and convenient.

1. *Heller's Test with Nitric Acid.*—Five to ten cc of nitric acid are poured into a test-tube, or, what is better, into a small conical glass (cognac glass), which is held at an angle, and carefully covered with an equal quantity of urine from a pipette. The urine must be slowly and carefully floated upon the acid (it is allowed to run from the pipette along the side of the glass) so that the two liquids

do not mix. If albumin is present, a sharply defined annular cloud is formed on the border between the two fluids. When a very small quantity of albumin is present, the ring is not formed for two to three minutes. The test depends upon the ability of nitric acid to quickly form acid-albumins, which are with difficulty soluble in an excess of acid. In this test the following sources of error are to be considered.

(*a*) In very concentrated urine a distinctly crystalline ring, composed of urea nitrate, is formed at the point of contact of the two fluids. With a little care it is very easy to distinguish this distinctly crystalline ring from the opaque, sharply defined albumin-ring. This precipitation of urea nitrate is prevented by diluting the urine.

(*b*) In urine containing a large amount of urates, an annular cloud is also formed, which, however, is distinguished from the albumin-ring by the fact that it is situated above the point of contact (of the two fluids), and disappears upon slight warning.

(*c*) Following the internal use of balsamic preparations (copaiba, balsam of Tolu and santal oil), a whitish ring is produced, due to colophonic acids. It is distinguished from the albumin-ring by the fact that its upper border is not distinct, and that it is soluble in alcohol.

(*d*) Urine containing nucleo-albumin shows an annular cloud with *Heller's* test. This ring, however, is not situated at the point of contact, but approximately in the middle of the urine layer; on shaking the ring dissolves.

(*e*) Since nitric acid oxidizes the urinary pigments, colored rings (red, brown, blue, green) are formed in every urine during this test, which can never be confused with the albumin-ring, since the cloud is absent.

This test gives a positive reaction with a dilution of

1 in 30,000—i.e., it detects 0.0033 per cent. albumin. With this amount of albumin the ring is formed only after two minutes. If the above-mentioned sources of error are borne in mind this test is very accurate and reliable.

2. *Boiling with Sodium Chloride and Acetic Acid.*—Five to eight cc of urine are treated in a test-tube with an equal quantity of a saturated solution of sodium chloride, and after the addition of 3 to 5 drops of acetic acid boiled. If the solution becomes cloudy, or a precipitate is formed, albumin is present. In order to detect very slight clouding, it is well to fill two test-tubes with the urine and reagent, and to boil but one of them; the other is used for comparison. If they are both examined in a good light against a dark background, the slightest clouding on boiling can be detected. When considerable albumin is present, a precipitate is often formed before heating, since the albuminoid bodies are converted by the acetic acid into acid albumins, which are precipitated by the salt and acetic acid. Colophonic acids are also precipitated by this test, but are dissolved upon the addition of alcohol.

This test is more delicate than *Heller's*, and has the advantage over other boiling tests that the color of the urine remains unchanged, and, therefore, the slightest cloudiness can be seen, especially if it is compared with unboiled urine which has been treated with the same reagent. It is especially to be recommended for the general practitioner, since, in cases of necessity, it may be performed in a large spoon, without chemical apparatus, with ordinary vinegar and common salt.

3. *Test with Sulphosalicylic Acid.*—Five to ten cc of filtered urine are treated with 5 to 10 drops of a 20 per cent. solution of sulphosalicylic acid. In the presence of a small quantity of albumin the fluid becomes opalescent;

in the presence of a greater quantity a distinct cloudiness or a whitish, flaky precipitate is produced. The urine must always have an acid reaction; alkaline or amphoteric urine must be slightly acidified with acetic acid. Uric acid and urates are not precipitated by this test. Albumoses (with the exception of deutero-albumoses) are precipitated, but they dissolve completely upon heating, while the precipitate of albumin remains unchanged. Colophonic acids react with this test, as with the previous tests.

The test is very delicate; quantities of albumin from 0.0015 per cent. giving a positive reaction. To detect slight opalescence, the urine is examined in direct light against a dark background, and compared under the same conditions with a test-tube containing an equal quantity of clear, filtered urine.

Based upon numerous urine analyses, we can recommend this test as both reliable and delicate.

4. *Test According to Spiegler and its Modification According to Jolles.*

Spiegler's reagent consists of:

Hydrarg. perchlor.	8.0
Acidi tartarici	4.0
Glycerini	20.0
Aquæ dest.	200.0

If urine which has been rendered strongly acid with acetic acid is floated upon this reagent, a whitish ring is formed in the presence of albumin. This test is very delicate, and detects even 0.0002 per cent.; it is, however, unfortunately, not always reliable, as the delicacy of the reaction depends upon the quantity of chlorides contained in the urine. In the presence of small quantities of sodium chloride the test is not so delicate. To obviate this the following reagent has been suggested by *Jolles :*

Hydrarg. perchlor.	10.0
Acidum succinicum	20.0
Sodium chlorid	10.0
Aquæ dest.	500.0

The reaction is carried out with this reagent in the following manner: 5 cc of urine are shaken with 1 cc of 30 per cent. acetic acid and 4 cc of the reagent. In another tube the same amount of urine is shaken with 1 cc of acetic acid and 4 cc of water. In this second tube the mucin will be precipitated, and by comparison albumin can be detected in the first tube if present.

The test must be carried out exactly according to the instructions; when too little acetic acid is used mercury combinations (mercury-phosphorus, mercury-ammonia) may be precipitated. When urine contains iodine, a precipitate of iodide of mercury is formed with this test; this is soluble in alcohol (in contradistinction to albumin).

Numerous tests have been suggested for the detection of albumin at the bedside which can be carried out with easily transportable, solid reagents and without boiling. These reagent-tablets and reagent-papers are, to be sure, very convenient, but they are unreliable, and cannot, therefore, be recommended. It is never advisable in examining the urine for albumin to limit one's self to the use of one of the described tests, but to carry out at least two of them. We proceed, as a rule, in the following manner:

As a preliminary test we use *Heller's* test. If this yields a distinctly positive result—i.e., if a typical albumin-ring is formed at once—the urine contains a large quantity of albumin, which is confirmed by the boiling test or the sulphosalicylic acid test. If the ring is formed only after several minutes, merely a trace of albumin is present, which must be controlled by the sulphosalicylic acid test, or by boiling with sodium chloride and acetic acid. If with *Heller's* test no ring at all is formed, the urine may either be entirely free from albumin or contain only a faint trace. As control tests, the most delicate reactions—*Jolles*' test or the sulphosalicylic acid test—must be carried out. We recommend that the urine be heated following the sulphosalicylic acid test in order that there may be no confusion with albumoses.

3. Albumoses and Peptones

The work of *Stadelmann* and his scholars has shown that true peptones, as understood by *Kuehnes*, have not as yet been with certainty detected in the urine.[1] All the previous cases which have been placed under the head of peptonuria were, in all probability, cases of the excretion of albumoses. These latter are present in the urine usually in those diseased conditions in which there is rapid destruction of normal or pathological tissue; in wide-spread exudates containing many cellular elements, abscesses, and in different febrile diseases. When spermatic fluid is present in the urine, traces of albumose are detected, since this secretion contains albumose. The albumoses which appear in the urine are composed, usually, of a mixture of deutero- and hetero-albumose. A peculiar albumose—a variety of hetero-albumose—the so-called *Bence-Jones* albumose, is present in cases of multiple myeloma. Very small quantities of albumose cannot be detected with certainty in the urine; they must be first isolated by precipitation from a large quantity of urine. In urine rich in albumoses (and free from albumin) the presence of the albumoses is shown by the unusual behavior of the albumin tests.

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[1] *Ito* and *Kotosky* are said, recently, to have detected peptone-*Kuehne* in the urine. This has, however, not yet been confirmed by other authors.

(*a*) With the boiling test, the urine which has become clear on heating, becomes cloudy on cooling, and yields a flaky precipitate.

(*b*) With the sulphosalicylic acid test the clouding or precipitate disappears on heating and returns on cooling. When albumin is also present, the urine does not become entirely clear on heating, and must, therefore, be first freed from albumin (cf. below). For the definite detection of albumoses in the urine the following method is used:

Ten cc[1] of urine are rendered acid with a few drops of hydrochloric acid, precipitated with phosphotungstic acid, and centrifugalized; the fluid is poured off from the precipitate, the precipitate well mixed with absolute alcohol, and again centrifugalized. The washing with alcohol is repeated until the alcohol remains absolutely colorless. The precipitate is then dissolved in a small quantity of sodium hydrate. The now deep-blue solution is slightly heated until it is again decolorized. After the solution has cooled, the biuret reaction is carried out—i.e., a very dilute solution of copper sulphate is floated upon the alkaline liquid. A reddish-violet coloration is produced at the point of contact of the two solutions.

4. Method of Freeing the Urine from Albumin

1. The urine is treated with sodium acetate and with enough ferric chloride to color it blood-red. If the urine is markedly acid, sodium hydrate solution is added, until it becomes neutral or faintly acid; it is then heated to the

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[1] Urine containing albumin must first be freed from the albumin.

boiling-point, whereupon the albumin is precipitated in large flakes.

2. The acid urine (neutral or alkaline urine must first be slightly acidified) is heated to the boiling-point. If the albumin is not precipitated in large flakes, but the solution merely becomes cloudy, a few drops of acetic acid are carefully added, and it is heated a minute or two longer. If this does not cause precipitation of the albumin in coarse flakes, a few cc of a saturated solution of sodium chloride are added, and it is again heated to the boiling-point. The coagulation of the albumin depends, above all, upon the quantity of acetic acid added; this must, therefore, be added with great care. An excess is as harmful as too little. The filtrate must be clear, and must show no clouding upon the addition of sulphosalicylic acid (exception: urine containing albumoses).

5. Albumins which may be Precipitated by Cold Acetic Acid

These albumins are mostly the nucleo-albumins and the globulins. They are found mostly in the physiological, cyclical, and orthostatic albuminurias; especially in the latter. They may be found together with the ordinary albumin or in a urine free from albumin. According to *Langstein* they are never or very seldom found in the urine of children suffering from chronic nephritis, but, as a rule in orthostatic albuminuria, so that we may use this for differential diagnosis. Not infrequently they occur in the urine of acute nephritis, icterus, and amyloidosis.

They are tested for in the following way:

To 10 cc of the filtered urine are added 5 to 10 drops of a 30 per cent. acetic acid solution, well shaken and diluted with two or three times its volume of water. In the presence of albumins which are precipitable by acetic acid, cloudiness ensues. For the purpose of comparison we use another test-tube in which water was added to the urine in like quantity and look against a dark background.

6. Fibrin

Fibrin is either passed in a coagulated condition, or separates out after the urine has stood awhile, forming a flaky precipitate. It is insoluble in water, salt solutions, and in cold dilute acids and alkalies. Fibrin is converted by alkalies into a jellylike mass, which, on continued heating, is gradually dissolved; it is also dissolved by boiling with acids.

For the detection of fibrin in the urine, the suspected clot is collected on a filter and washed with a 1 per cent. sodium chloride solution until the wash-water is no longer alkaline in reaction. The residue is then digested and dissolved with a warm 5 per cent. soda solution or a 0.5 per cent. hydrochloric acid solution. The solution must give the characteristic albumin reactions. The easiest means of detecting fibrin is by its characteristic appearance under the microscope.

THE CARBOHYDRATES OF THE URINE

Normal urine contains usually only a very small quantity of carbohydrates; animal gums and a faint trace of glucose, which cannot be detected by the ordinary reactions. Under abnormal and pathological conditions, in addition to glucose, the following varieties of sugar may be found: Lactose, maltose, inosite, and pentose. In the clinical examination of the urine, glucose especially comes into consideration.

7. Glucose, $C_6H_{12}O_6$ (Grape-Sugar, Dextrose)

The detection of glucose in the urine depends upon the following facts:

1. In alkaline solutions glucose has a tendency to absorb oxygen and therefore acts as a strong reducing agent. Metallic oxides are reduced by it to protoxides or to pure metal.

2. If a solution of glucose is treated with yeast, alcoholic fermentation takes place, by which glucose is decomposed, principally into alcohol and carbon dioxide, $C_6H_{12}O_6 = 2C_2H_6O + 2CO_2$.

3. Glucose combines with phenylhydrazin in the presence of sodium acetate forming a crystalline product: Phenylglucosazon, $C_6H_{12}O_6 + 2NH_2 - NH. C_6H_5 = C_6H_{10}O_4 : (N - NHC_6H_5)_2 + 2H_2O + H_2$.

4. Its watery solution rotates the plane of polarized light to the right, and in fact +52.5°. Freshly prepared solutions show bi-rotation, which is removed by heating or long standing.

SUGAR REACTIONS WHICH DEPEND UPON THE REDUCING PROPERTY OF GLUCOSE

1. ***Nylander's Test.***—A colorless, alkaline solution of bismuth oxide becomes black when heated with glucose, since the bismuth oxide is converted into bismuth protoxide or metallic bismuth. Nylander's reagent has the following composition:

Bismuth subnitrite	2.0
Rochelle salts	4.0
Sodium hydrate	10.0
Aqua dest.	100.0

Performance of the Test.—Five to ten cc of urine are treated with 15 to 20 drops (more does no harm) of the reagent, and boiled two minutes (not less). In every urine a whitish flaky precipitate of phosphates is formed, which, if the urine contains no sugar, remains unchanged. In urine containing sugar, first the precipitate, and then the entire liquid, becomes yellowish-brown and finally black. When small quantities of sugar are present (under 0.1 per cent.) a distinct black coloration of the precipitate cannot be seen during the boiling; the coloration appears only after the precipitate has settled. In such cases the liquid is merely colored dark yellow or dark brown. The solution must be carefully heated, in order that it may boil quietly without marked sputtering. To accomplish this, the test-tube is removed from the upper hot portion of the flame as soon as the liquid begins to bubble, and held in the cooler lower portion during the remainder of the boiling. *Nylander's* test is very sensitive; therefore when the result is negative it can be assumed with certainty that the urine is absolutely free from sugar.

A positive result, however, does not always mean the presence of glucose, since other substances, which may be present in the urine, can give the same reaction. The most important of these substances are the following:

(*a*) *Albumin.*—In the presence of less than 0.2 per cent. of albumin a reddish-brown coloration is produced; in the presence of larger quantities of albumin a blackish-brown coloration, which may be confused with bismuth, reduced by sugar. The black coloration in urine, containing albumin, is due to the decomposition of the albumin and the formation of bismuth sulphate. It is well, therefore, to free the urine from albumin, according to one of the methods suggested, before carrying out the test. There are also cases in which, in spite of the presence of sugar, reduction does not take place, since the entire reagent has combined with albumin. This, however, does not happen if sufficient reagent is used.

(*b*) *Chrysophanic acid*, which is excreted in the urine following the use of preparations of rheum and senna, also reduces *Nylander's* reagent. Its presence is betrayed by a reddish coloration of the urine upon the addition of the reagent. This coloration resists the addition of sodium hydrate, but disappears absolutely upon the addition of acetic acid (in contradistinction to hæmoglobin).

(*c*) *Salol*, *antipyrin*, *menthol*, and *turpentine oil* are excreted in the urine after internal use, in combination with glycuronic acid, and faintly reduce *Nylander's* reagent.

When the urine is markedly decomposed, due to fermentation, and contains a large quantity of ammonium carbonate, the reaction may be negative even in the presence of sugar, since the sodium hydrate of the reagent reacts with the ammonium carbonate, forming sodium carbonate and ammonia; the latter is driven off by the boiling, so that the strong alkalinity necessary for the reduction is no longer present.

2. **Trommer's Test.**—This depends upon the following facts:

If copper sulphate is added to a solution of sodium hydrate, a precipitate of copper hydroxide is formed, which is insoluble in sodium hydrate, $2NaOH + CuSO_4 = Na_2SO_4 + Cu(OH_2)$. This precipitate is, however, soluble in tartrates, ammonia, albumin, uric acid, creatinin, and glucose. Normal urine contains a small quantity of some of these copper hydroxide solvents.

If an alkaline blue solution of copper hydroxide is heated, it remains unchanged if the solution contains no reducing substances.

If, however, such substances are present, they form copper protoxide from the copper hydroxide, the liquid loses its blue color, becomes yellow or colorless, and a yellow or red precipitate is formed. The yellow precipitate consists of cuprous hydroxide, the red of pure anhydrous cuprous oxide.

Procedure.—Five to eight cc of urine are treated with about a quarter as much potassium or sodium hydrate (10 per cent.), and, with vigorous agitation, a 10 per cent. solution of copper sulphate added, drop by drop, until a small amount of the copper hydroxide which is formed, remains undissolved. The mixture is now heated, best at the surface of the liquid, to the boiling-point. If sugar is present, a yellow clouding is produced at the portion heated, which, without further heating, quickly spreads throughout the entire liquid, and a yellow or red finely granular precipitate is thrown down.

The reaction behaves in this manner only in diabetic urine.

When but a small amount of sugar is present (under 0.5 per cent.) the liquid turns yellow, but frequently no precipitate of cuprous oxide is formed. On the other hand, the liquid may turn yellow, and even (after cooling) a late precipitation of cuprous oxide may be produced in concentrated urine, though it is absolutely free from sugar. This atypical behavior of the urine with *Trommer's* test is due to the following facts:

(*a*) The urine contains substances which hold cuprous oxide in solution (uric acid, creatinin, ammoniates).

(*b*) Normal urine contains substances which reduce cupric oxide (uric acid, glycuronic acid, carbohydrates, etc.).

The solvent property of normal urine for cuprous oxide is so great that frequently, if normal urine is treated with 0.5 per cent. glucose and *Trommer's* test is carried out, no precipitation of cuprous oxide is produced.

In diabetic urine the precipitation of cuprous oxide may occur in the presence of less sugar, since the solvents of cuprous oxide are relatively decreased owing to the polyuria, which is, as a rule, present.

At any rate, *Trommer's* test frequently leaves the examiner in doubt as to the presence of sugar in the urine, and must, therefore, be considered as an unreliable and inaccurate test. It is of value to the practitioner only in that in pronounced cases the quantity of sugar may be roughly estimated from the volume of cuprous oxide precipitated. For the more exact qualitative detection of sugar, *Trommer's* test should be used only in the following improved form:

3. **Fehling's Test.**—Necessary reagents:

(*Fehling* No. 1.)

1. A 7 per cent. solution of copper sulphate.

(*Fehling* No. 2.)

2. Sodium hydrate	10.0
Sodium potassium tartrate (Rochelle salts)	35.0
Aqua dest.	100.0

Procedure.—Ten drops of each of these solutions are placed in a test-tube, the mixture shaken, diluted with three times as much water, and boiled. Three to five drops of the urine to be examined are then added to the hot solution, and it is again boiled; if no sugar is present, the solution keeps its blue color. If considerable sugar is present, a yellow or yellowish-red coloration of the solution, and a liberal, finely granular, precipitate of cuprous oxide are produced as soon as the urine is added. If less sugar is present, the alteration of color and the precipitation of cuprous oxide do not take place until the solution has been boiled again.

The solvent action of normal urine on cuprous oxide and also its reducing power are negligible, owing to the small amount of urine employed. By the presence of the sodium potassium tartrate in the reagent, the precipitation of cupric hydroxide which, on boiling, forms black oxide of copper and often obscures the reaction in *Trommer's* test, is entirely prevented, since the Rochelle salts hold the cupric hydroxide in solution.

4. ***Fermentation Test.***—A piece of fresh compressed yeast, the size of a pea, which must be absolutely free from sugar, is rubbed in a test-tube with a small quantity of urine (1 to 2 cc), and 20 to 25 cc of urine are added to this paste. If the urine is alkaline in reaction, it is acidified with tartaric acid. *Einhorn's* saccharometer (Fig. 23) is filled with this urine in such a manner that tube *a*

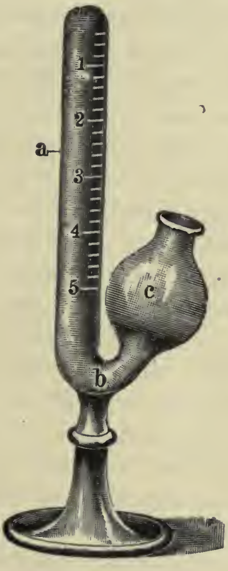

FIG. 23

is completely filled, to the entire exclusion of air, and bulb *c* half-filled, and is set aside in a moderately warm place (25 to 30° C.). The horizontal portion of the apparatus *b* may be closed with a few drops of mercury, but this is not necessary for the qualitative test. In the presence of sugar, even after a few hours, carbon dioxide collects at the top of tube *a*, since the sugar is decomposed by fermentation into alcohol and carbon dioxide. If sugar is absent, no gas is formed. The test should not be considered as completed before the end of twenty-four hours. As commercial yeast is not always free from sugar and may, therefore, be fermentable, two controls should be made at the same time: one saccharometer should be filled, in the manner described, with water acidified with tartaric acid and yeast; the other with an acidified glucose solution (0.5 per cent.) and yeast. The fitness of the yeast for use is established, if no gas forms in the first control saccharometer, while a large amount of carbon dioxide forms in the second. If proof is desired that the gas produced is really carbon dioxide, a small amount of sodium hydrate solution may be introduced into tube *a* by means of a curved pipette. If the gas bubble disappears, it was composed of carbon dioxide. The fermentation test is the only absolutely sure and exact method for the detection of sugar in the urine, since no other normal or pathological constituent of the urine gives a similar reaction. It is at the same time sufficiently delicate. The presence of sugar, from 0.05 per cent. on, is distinctly detected by means of this test.

5. ***The Phenyl-Hydrazin Test (According to Kowarsky).***— Five drops of pure phenyl-hydrazin (*phenyl-hydrazinum purum*) are treated in an ordinary test-tube with 10 drops of acetic acid and the mixture lightly shaken. About 15 drops of a saturated solution of sodium chloride are then

added, whereupon the mixture congeals to a paste. About 10 cc of urine are added to this paste, and heated carefully over a flame. The solution is boiled at least two minutes. On slowly cooling, a yellow precipitate, consisting of typical crystals of phenyl-glucosazone, is thrown down. The rapidity of the precipitation depends upon the amount of sugar in the urine. In the presence of more than 0.2 per cent. of sugar the precipitation is formed in a few minutes; in the presence of less, often not for five minutes to half an hour. This test is very delicate and detects sugar from 0.03 per cent. onward.

Opinions are as yet divided as to the practical value of the phenyl-hydrazin test; on the one hand, it is claimed that normal urine contains substances which combine with phenyl-hydrazin to form osazone and produce similar crystals; on the other hand, it is considered as reliable, and as furnishing positive evidence in doubtful cases.

The fact is that normal urine frequently contains substances (combinations with glycuronic acid) which produce similar crystals with this test. These substances are, however, present in very small quantity, and with a certain amount of practice are easy to distinguish from the typical glucosazone crystals, since they are plumper and thicker than the true crystals, and not so typically arranged. In addition to the glycuronic acid compounds the pentoses also form osazone. As yet, however, but a few cases of true pentosuria have been reported in the entire literature, so that these carbohydrates are of little importance in considering the phenyl-hydrazin test. Based upon our own experience, which covers many thousands of urine analyses (in which the result of the phenyl-hydrazin test has always been controlled by the fermentation test), we consider this test very delicate and reliable, and can recommend it especially in doubtful cases.

Concerning the detection of sugar by polarization, cf. the Quantitative Examination of the Urine.

8. Lactose, $C_{12}H_{22}O_{11}$ (Milk-Sugar)

Lactose is present in small amount (maximum 1 per cent.) in the urine of women when there is stagnation of milk. Like glucose, it possesses the property of reducing metallic oxides in alkaline solution, and of rotating the plane of polarized light to the right. It is not, however, subject to alcoholic fermentation by yeast. On boiling with dilute acids it is converted into glucose and fermentable galactose. The latter also forms a crystalline combination with phenyl-hydrazin (galactosazone). According to *Rubner*, lactose is detected in the urine by the following test:

Ten cc of urine are boiled three to four minutes with a large quantity of lead acetate; in the presence of lactose the solution becomes yellow or brown; ammonia is added to the hot solution as long as the precipitate is dissolved. The solution first assumes an intense brick-red color, then throws down a beautiful cherry-red to copper-colored precipitate, and becomes colorless. The reaction is not delicate, and only detects positively 0.3 to 0.5 per cent. or more of lactose. If it is of especial importance to detect lactose, it must be isolated from a large quantity of urine and tested in its pure state.

9. Levulose, $C_6H_{12}O_6$ (Fruit-Sugar)

Levulose rarely appears in the urine, but when present is usually accompanied by glucose. It differs from glucose in that it rotates the plane of polarized light to the left. Its behavior toward the metallic oxides, yeast, and phenylhydrazin is identical with that of glucose. For the detection of levulose in the urine the sugar present must be estimated by at least two methods—by polarization and fermentation, or by polarization and titration according to *Fehling*. Levulose is detected if the urine rotates the plane of polarized light less to the right than corresponds to the amount of sugar present, estimated by another exact quantitative method. It must, however, also be determined that there are no other lævorotary substances (albumin, β-oxybutyric acid, etc.) present in the urine.

Seliwanoff has suggested the following color reaction for the detection of levulose: A solution of levulose is heated with resorcin and hydrochloric acid, a precipitate is formed which dissolves in alcohol with the production of a red color. This reaction is not very accurate.[1] For positive determination levulose must (like lactose) be isolated and tested in its pure state.

10. Pentose, $C_5H_{10}O_5$ (Pentaglucose)

Pentoses differ from other varieties of sugar in that they are unfermentable. They reduce *Fehling's* solution only after long heating, and only very faintly reduce *Nylander's* reagent. Pentoses are best detected in the urine by means of the orcin test, which is carried out in the following manner:

Five to eight cc of fuming hydrochloric acid are slightly supersaturated, under heating, with orcin (a knife-point of orcin is sufficient). One to two cc of urine are added to the hot solution, and it is again heated to the boiling-point. If pentoses are present the solution turns green. The pigment is extracted with a small amount of amyl alcohol and examined spectroscopically. An absorption band is seen in red between C and D. The orcin test depends upon the formation of furfurol, which when boiled with orcin and hydrochloric acid produces a green pigment.

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[1] According to *Loew* pyrogallol gives a similar reaction.

11. Glycuronic Acid ($CHO\,[CHOH]_4\,COOH$)

According to its chemical composition glycuronic acid is closely related to the carbohydrates. It is considered as the first product of the oxidation of glucose. Free glycuronic acid does not appear in the urine; it is excreted usually as a double compound with phenyl, skatol, indoxyl, thymol, etc., both in normal and pathological urine. It comes into consideration in the clinical examination of urine only in that it gives certain reactions which resemble those of glucose, and may often be confused with it. Free glycuronic acid rotates the plane of polarized light to the right, combined glycuronic acid to the left. The slight lævorotary action of normal urine is in all probability due to the presence of glycuronic acid. This may be established by the fact that after boiling with dilute acids (sulphuric acid) the urine shows a dextrorotary action, since glycuronic acid is freed by boiling with acids. It slightly reduces *Fehling's* and *Nylander's* reagents. Glycuronic acid compounds are precipitated by lead acetate (in contradistinction to glucose). The reducing action and the lævorotary action are not, however, positive proof of the presence of combined glycuronic acid. For the positive detection of combined glycuronic acid the following procedure must, according to *Salkowski* and *Paul Mayer*, be carried out. The phloroglucin test is first carried out.

Five to six cc of fuming hydrochloric acid are saturated with phloroglucin while hot, so that on cooling a small excess of the latter remains undissolved, and this solution is divided into two portions, to one of which, after cooling, ½ cc of the urine to be examined is added, and to the other the same amount of normal urine of nearly equal concentration. If both tubes are plunged into a beaker of boiling water, the urine containing glycuronic acid assumes an intense red color, which gradually spreads from above downward. The normal urine does not change its color, or changes it very slightly. As pentoses also give this reaction, it must be determined that an untreated sample of the urine gives a negative result with the orcin test. This latter reaction must, however, be distinctly given, after boiling with acids, by urine containing combined glycuronic acid.

Fifty cc of urine are treated with sufficient concentrated sulphuric acid to make the solution correspond to a 1 per cent. solution of sulphuric acid, and heated in a porcelain dish over a free flame. The exact length of heating cannot be given. It is usually sufficient to keep the urine boiling for one to three minutes. The orcin test is then carried out directly with the solution without filtering it. If the reaction does not take place at once, the solution must be again boiled for one to two minutes.

12. Acetone, Diacetic Acid, β-Oxybutyric Acid

ACETONE, $CH_3.CO.CH_3$.

Normal urine contains but a very faint trace of acetone, too slight to be detected by the ordinary reactions (at the most 0.01 gramme in the daily output). Under abnormal and pathological conditions (diabetes, fever, exclusive meat diet, starvation, disturbances of digestion) the amount of acetone in the urine may reach 0.5 or even 1.0 gramme in twenty-four hours.

Detection.—*Legal's Test.*—Eight to ten cc of urine are treated with 3 to 5 drops of a freshly prepared saturated solution of sodium nitroprusside, and rendered alkaline with a few drops of sodium hydrate. On the addition of the sodium hydrate, a ruby-red coloration appears in almost every urine, and is due to a normal constituent of the urine, creatinin. If the red solution is supersaturated with concentrated acetic acid, the red color becomes more intense in the presence of acetone, and passes into crimson, while if acetone is not present the red color entirely disappears. The reaction is not very delicate: according to *von Jaksch*, only quantities over 0.8 milligramme are detected. To detect smaller quantities the acidified urine (100 cm^3) must be distilled and the first portion of the distillate must be examined with the more delicate.

Lieben's Iodoform Test.—Five to ten cc of the distillate are treated with *Lugol's* iodine potassium iodide solution, and sodium hydrate. In the presence of acetone, iodoform is produced which may be easily recognized by its odor and its crystalline forms (hexagonal and stellate plates). This reaction is much more delicate than *Legal's;* it has, however, the disadvantage that alcohol and aldehyde also give this reaction, and alcohol (due to fermentation of the sugar) may be present in precisely those cases in which the detection of acetone is of the greatest importance—namely, in diabetic urine.

DIACETIC ACID, CH_3COCH_2COOH

Diacetic acid is almost never present in the urine, except under pathological conditions. It is very frequently accompanied by acetone and β-oxybutyric acid. It is formed from β-oxybutyric acid, and decomposes easily into acetone and carbon dioxide. The urine must, therefore, be examined in as fresh a condition as possible.

Detection.—*Gerhardt's Test.*—Five to ten cc of urine are treated with a solution of ferric chloride as long as a sediment is formed. In the presence of diacetic acid a Bordeaux-red coloration is produced. If the red color of the solution is not distinct, it is well to remove the precipitate of ferric phosphate by filtration. The urine gives a very similar reaction following the internal use of salicylic acid, antipyrin, thallin, phenacetin, and certain other drugs. A positive result of the reaction must, therefore, be confirmed by a control test. Five to ten cc of the urine are boiled three to five minutes, and, after cooling, are treated with ferric chloride in the above-described manner. If the positive result was due to diacetic acid the red color will not appear, since diacetic acid is decomposed by boiling; if it was due to drugs, the red color, upon the addition of ferric chloride, will appear after boiling as well as before.

β-OXYBUTYRIC ACID, $CH_3CH(OH)CH_2COOH$

This acid appears in the urine in severe cases of diabetes and is always accompanied by acetone and diacetic acid, which are considered as products of its decomposition. It rotates the plane of polarized light to the left, and can, therefore, influence the quantitative estimation of glucose by polarization, or even render it impossible.

According to *Kuelz*, β-oxybutyric acid is detected in the urine in the following manner:

The urine is fermented with yeast, and most of the lævorotary substances—with the exception of β-oxybutyric acid—are precipitated with lead acetate and ammonia. The filtrate is examined with the polarimeter. Rotation to the left suggests the presence of the acid.

13. The Pigments and Chromogens of the Urine

A. INDICAN (INDOXYLSULPHURIC ACID), $C_8H_6NOSO_2OH$

Normal human urine contains but a small quantity of indican—on the average, 0.06 gramme in twenty-four hours. Under pathological conditions, ten to fifteen times this quantity may be present. Indol, from which indican is derived, is formed in the intestines as a product of the decomposition of albumin. After its absorption indol is oxidized to indoxyl, and then combines with the sulphuric acid of the urine, and is thus excreted as indoxylsulphuric acid. Indican in the urine may be decomposed by mineral acids and the indoxyl converted by oxidation into indigo. Under pathological conditions there may be a spontaneous excretion of indigo in the urine, in which case it may be either in solution or in the sediment.

Detection.—One-third of a test-tube of urine is treated with an equal quantity of concentrated hydrochloric acid, 15 drops of chloroform, and 2 to 3 drops of a 2 per cent. potassium permanganate solution. The test-tube is corked and repeatedly inverted, whereupon the indigo-blue which is formed is extracted by the chloroform, coloring the latter distinctly blue. It must not be vigorously shaken, since chloroform forms an emulsion with the urine which it is very hard to decompose.

The reaction depends upon the decomposition of the indican by hydrochloric acid, and the oxidation of the freed indoxyl, by potassium permanganate to indigo-blue. The potassium permanganate must be added very carefully; at first, not more than 2 to 3 drops are added, since by too strong oxidation the indigo-blue can be at once further oxidized to yellow isatin, and thus be entirely overlooked. If the permanganate solution is further added a drop at a time, it will be noticed that in urine containing but little indican the blue coloration of the chloroform disappears after but a few drops, while in urine rich in indican the blue coloration becomes more intense as the solution is added, and a comparatively large amount of the solution must be added before the indigo-blue is entirely converted into isatin. This procedure may be used as a method for the quantitative estimation of indican.

Not infrequently in the indican test a rose-red coloration of the chloroform is produced, instead of a blue coloration. This is the case following the internal use of preparations of iodine. The iodine is freed from its combinations by the hydrochloric acid and the oxidizing material, and causes the red coloration of the chloroform. To offset this a crystal of sodium thiosulphate is added, and the solution shaken. The iodine then forms colorless iodides, and the chloroform is decolorized. In the presence of both iodine and indican a violet coloration of the chloroform is produced, which, on treating with sodium thiosulphate, becomes blue.

In the presence of chrysophanic acid a greenish-yellow coloration of the chloroform is produced with the indican test.

A yellowish coloration is produced following the internal use of bromine preparations.

B. UROBILIN AND UROBILINOGEN

Normal urine contains a very small quantity of urobilin in the form of a chromogen—urobilinogen—which by the action of light, and by the presence of acids, is very easily converted into the pigment. In its pure state urobilin is an amorphous, reddish-brown substance, soluble with difficulty in water. It is readily soluble in alcohol, chloroform, and alkalies. It forms insoluble salts with the alkaline earths and the heavy metals.

Detection by the Schlesinger Method.—Ten to fifteen cc of urine are treated with an equal quantity of 10 per cent. alcohol solution of zinc acetate, and filtered. The filtrate when held against a dark background shows a distinct greenish fluorescence. This test is very delicate.

The test for **Urobilinogen** by *Ehrlich's* aldehyd reaction. A few drops of a 2 per cent. solution of dimethyl-paramido-benz-aldehyd in 20 per cent. HCl are added to a few cc of urine and, should the reaction not appear soon, a little HCl concentrated is added. Most urines are colored slightly red by this. In the presence of an increased quantity of urobilinogen the red color becomes quite intense and is clearly demonstrable even after several dilutions; spectroscopic analysis shows a broad absorption band between *Frauenhofer's* lines D and E. Urobilin does not give this reaction.

C. Biliary Pigments

Normal urine contains no biliary pigments. Under pathological conditions these gain entrance to the circulation, and thence to the urine. Bilirubin is the only one of the biliary pigments which has with certainty been detected in fresh icteric urine. The others—biliverdin, biliprasin, bilifuscin—are formed only after the urine has stood a long time, and must therefore be considered as derivatives of bilirubin.

Icteric urine is saffron-yellow to reddish or dark-brown in color. On shaking, a characteristic yellow foam is produced.

Detection.—(*a*) *Gmelin's Test.*—Five to six cc of ordinary concentrated nitric acid are treated with a few drops of yellow, fuming nitric acid, and carefully covered with an equal quantity of the urine to be examined. In the presence of biliary pigments an emerald-green ring is formed at the point of contact of the two liquids, beneath which a blue, violet, or yellow ring is gradually formed. The test should be considered positive only when the green ring is very pronounced, since the blue and violet rings may be caused by the oxidation of other substances which appear in the urine (indican, indigo red). This test is not delicate; it detects the presence of only 5 per cent. or more of bile.

(*b*) *Modification According to Rosenbach.*—A large quantity of urine is filtered through a single filter-paper, and the inner side of the paper is touched with a drop of nitric acid which contains nitrous acid (prepare as in test *a*). The colors are then produced as in test *a*.

This test is somewhat more delicate than *Gmelin's* test, and is more distinct in the presence of small amounts of bile-pigment. It must be remembered, however, in this test that a green ring may be produced following the use of antipyrin. Not rarely a blue color is produced, following the ingestion of preparations of iodine, since the iodine is freed by the nitric and nitrous acids, and, combining with the starch present in the filter-paper, causes a more or less intense blue coloration. This blue color may absolutely obscure the green color of the bile-pigment.

(*c*) *Test with Tincture of Iodine According to Rosin.*—The urine (10 to 15 cc) is covered in a test-tube with a dilution of 1:10 of the official tincture of iodine. A green ring, which lasts for hours, is formed at the point of contact of the solutions. The test is more delicate than *Gmelin's*.

(*d*) *Huppert's Test.*—One hundred cc of urine are rendered distinctly alkaline with sodium carbonate, and the biliary pigments are precipitated with an excess of barium chloride or barium hydrate. The yellow precipitate is collected on a filter, and boiled with alcohol, to which a few drops of diluted sulphuric acid have been added. The precipitate is decolorized by this procedure, while the solution assumes a beautiful green color. After this alcoholic solution has been diluted with an equal quantity of water, the pigment can be extracted with a few cc of chloroform. The chloroform becomes deep green. This is the surest and most delicate method for detecting biliary pigments in the urine.

D. Blood Pigment, Hæmoglobin

The following varieties of hæmoglobin are distinguished:

1. Oxyhæmoglobin.
2. Methæmoglobin, contains the same amount of oxygen as oxyhæmoglobin, but in more stable combination.

Oxyhæmoglobin.	Methæmoglobin.	Reduced Hæmoglobin.
Soluble in water, coloring it bright-red; insoluble in alcohol; crystallizable; easily decomposed in watery solutions. On heating these solutions, a brownish precipitate is formed even at 70° C., which is composed of albumin and hæmatin. Dilute solutions are yellowish-red in color, and show spectroscopically (0.01 per cent.) characteristic absorption bands. It is also decomposed into hæmatin and albumin by the action of acids and alkalies.	Solutions brown in color; crystallizes in the form of brown needles and plates; is easily formed from oxyhæmoglobin by the action of acids and acid salts; hence its presence in the urine. In acid or neutral solutions, it shows, in addition to the absorption bands of oxyhæmoglobin, two bands, the first of which is more pronounced than the others.	In moderately dilute solutions, greenish to brownish-red; is formed from oxyhæmoglobin and methæmoglobin by the action of reducing substances; for example, by the addition of a few drops of ammonium hydrosulphide or stannous chloride in ammoniacal solution. Shows only one broad absorption band. On shaking with air, it is converted into oxyhæmoglobin; with acetic acid and a trace of sodium chloride, it forms hæmin (hæmatin chloride, dark-brown rhomboid plates).

3. Reduced hæmoglobin, contains less oxygen, and is formed by reduction of the two previous.

4. Carbon-monoxide hæmoglobin.

5. Prussic acid methæmoglobin.

In examining the urine, especially the first three varieties of hæmoglobin come into consideration; we give, therefore, in the above table a summary of their physical and chemical properties.

All varieties of hæmoglobin are albuminoid substances, and, therefore, when they are present, the urine gives the various reactions for albumin.

The urine may contain the various constituents of the blood, hæmoglobin, red blood-corpuscles, fibrin (hæmaturia), or only the pigment (hæmoglobinuria). The presence of red blood-corpuscles and fibrin is detected microscopically; that of pigment by the following reactions:

1. ***Heller's Test.***—This reaction depends upon the formation of hæmatin by the action of sodium hydrate. The hæmatin is taken up by the earthy phosphates simultaneously with their precipitation. Ten to fifteen cc of urine are rendered strongly alkaline with sodium hydrate, and heated to the boiling-point; a flaky, red precipitate is produced. In the presence of a small quantity of hæmoglobin the color becomes distinct only after the precipitate has settled.

If no precipitate is formed on heating (due to the absence of earthy phosphates), the urine is treated with an equal quantity of normal urine and the test repeated.

Following the use of rheum, senna, cascara sagrada, and santonin, the urine gives a similar reaction. Urine containing hæmoglobin is distinguished, however, by the fact that on the careful addition of acetic acid only a portion of the precipitate is dissolved—namely, the phosphates—while the hæmatin remains in reddish-brown flakes. If the positive reaction was due to drugs, the sediment and the coloration disappear absolutely upon the addition of the acid.

2. **Almen's Test** depends upon the transportation of oxygen from turpentine to guaiacum resin by hæmoglobin, and the consequent oxidation of the guaiacum.

Ten to fifteen cc of urine are covered with an emulsion of equal parts of tincture of guaiacum and old (ozonized) turpentine. At the point of contact of the solutions a ring is formed (due to the precipitation of resin) which is at first white, but, in the presence of hæmoglobin, soon assumes a beautiful blue color. The test is, to be sure, more delicate than *Heller's*, but it is little suited for the detection of hæmoglobin in the urine, since animal cells, especially pus-corpuscles, may cause a similar reaction.

3. The **Benzidin Test** is executed in the same way, as in the examination of the stomach contents.

4. **Spectroscopical Examination.**

Principle.—Each variety of hæmoglobin possess the property of absorbing certain rays of light, so that dark stripes are formed in the spectrum (absorption bands), which are characteristic of that variety. The best and most convenient spectroscopes for examining the urine are *Browning's* or *Vogel's* pocket spectroscopes. They show the absorption phenomena even more distinctly and sharply than the majority of the larger forms of apparatus. The determination of the position of the absorption bands in the spectrum is made in these forms of apparatus by comparison with the solar spectrum, which is simultaneously shown by means of a special arrangement (comparison prism). For spectroscopical examination the urine is filtered, and diluted as necessary; alkaline urine is acidified with acetic acid. The urine is then poured into a receptacle having two parallel sides of colorless glass (hæmatinometer);[1] this is held against the opening of the spectroscope so that the rays of light (from a gas or oil flame or daylight) pass through the urine perpendicularly to the sides of the glass. In observing the spectrum the position of the absorption bands is determined by comparison with the solar spectrum, which, by means of a simple arrangement, may be thrown in and out of focus. The characteristic spectra of the varieties of hæmoglobin which come into consideration in the examination of urine can be seen in the table.

E. HÆMATOPORPHYRIN

Hæmatoporphyrin may be made from hæmatin artificially, by the action of concentrated sulphuric acid, and the treatment of the solution with acid alcohol and stannum, or zinc. Hæmatoporphyrin differs from hæmatin only in that it contains no iron. Hæmatoporphyrin has been detected in the urine in various diseases. It also very frequently appears following the use of large quantities of sulphonal and trional. Urine containing hæmatoporphyrin is brownish-red, in thin layers yellowish-red.

Detection.—Twenty to twenty-five cc of urine are precipitated with a mixture of equal parts of a saturated solution of barium hydrate and of a 10 per cent. solution of barium chloride, the precipitate collected upon a filter, and washed with water, and once with alcohol. The precipitate is then rubbed with a few drops of hydrochloric acid and a small quantity of alcohol, allowed to stand awhile, then heated on a water-bath and filtered. The acid, red filtrate shows, on spectroscopical examination, two absorption bands: the first in front of D, the second, broader band, between D and E.

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[1] An ordinary test-tube may be used instead of the hæmatinometer.

If the solution is rendered alkaline with ammonia, it assumes a yellow color, and shows, spectroscopically, four bands from red to violet. The first and third bands are narrow, the second and fourth wide.

F. Melanin

Normal urine contains no melanin. Melanuria is a pathological phenomenon, and appears in patients having melanotic tumors. Freshly passed urine contains probably only the chromogen, melanogen, which is later converted, by oxidation, into melanin. Urine containing melanin is dark in color, and on standing exposed to the air becomes dark brown to black.

Detection.—If a solution of ferric chloride or potassium bichromate is added to the urine acidified with dilute sulphuric acid, a dark coloration is produced. The same coloration is produced by the addition of chlorine or bromine water to such acidified urine. An excess of the oxidizing agent decolorizes the urine with the production of a dirty yellow precipitate.

14. Diazo Reaction

The substances which cause this reaction, which was suggested by *Ehrlich*, are as yet unknown. Normal urine does not give the reaction; it appears only in the urine in febrile diseases, most often in typhoid fever, tuberculosis, and measles.

For the performance of the test two solutions are necessary:

1.	Sodium nitrite	0.5
	Aquæ dest.	100.0
2.	Acidum sulphanilicum	5.0
	Acidum hydrochloricum	50.0
	Aquæ dest.	1,000.0

Two cc of the first and 98 cc of the second solution are mixed. The reaction is carried out in the following manner:

Ten to fifteen cc of urine are treated in a test-tube with an equal quantity of the reagent, shaken vigorously until a foam is produced, and then about 1 cc of ammonia is added. The reaction is positive if the foam and liquid are both colored brilliant red. Normal urine is only colored yellow with this test. After twenty-four hours' standing, a positive test throws down a precipitate, the upper portion of which is blue, green, or black.

The urine gives a similar reaction following the internal use of naphthaline. While following the use of preparations of tannic acid the previously pronounced reaction disappears entirely.

15. Adventitious Constituents of the Urine

Of the great number of adventitious constituents of the urine, the majority of which follow the ingestion of drugs, only those will be considered here which in the first place are easy to detect, and in the second have a certain clinical or therapeutic significance.

1. **Mercury** (detection according to *Stukowenkoff*).—Five cc of egg-albumin are thoroughly rubbed in a mortar with an equal quantity of a saturated solution of sodium chloride, and dissolved in 500 cc of urine. The solution is then warmed on a water-bath until the albumin is completely coagulated. The precipitate is collected on a filter, dried between filter-paper, and then rubbed in a mortar with about 10 cc of concentrated hydrochloric acid. Forty cc of hydrochloric acid are then added, and the solution, in which a copper or brass spiral is placed, is allowed to stand twenty-four hours in a glass beaker or cylinder. The albumin and the mercury which has been collected by

it are dissolved by the hydrochloric acid. The mercury forms an amalgam on the surface of the copper spiral. The spiral is washed, first with cold then with hot water, rinsed in alcohol and ether, and dried in the air. It is placed in a dry piece of narrow glass tubing, which has been sealed at one end by melting. A crystal of iodine is then sublimed, by slight heating, at the upper end of the spiral. The tube is carefully heated with continuous turning from the lower to the upper end of the copper spiral. The mercury is thus sublimed, and, combining with the iodine, forms a brick-red ring of mercuric iodide. The width of the ring is, if the instructions are carried out exactly, proportional to the quantity of mercury, and a quantitative estimation is thus rendered possible. It is only necessary to have a scale—that is, a series of mercuric iodide rings obtained from definite quantities of mercury (1, 2, 3, 4, etc., milligrammes)—and to compare the ring obtained with this scale.

This method is very delicate; 0.0005 gramme of mercury can be clearly detected. If the ring is not clearly seen macroscopically, the characteristic red crystals of mercuric iodide can be easily detected microscopically with low power.

2. **Arsenic** in the urine is detected, according to *Gutzeit*, in the following manner: One cc of urine is treated in a glass cylinder or wide test-tube with 4 cc of dilute sulphuric or hydrochloric acid, and a piece of arsenic-free zinc. The receptacle is closed with a cotton plug, and covered with filter-paper moistened with a concentrated solution of silver nitrate. The filter-paper assumes a lemon-yellow color, which on longer standing turns black, due to the formation of metallic silver (from the yellow arsenate of silver). In the urine this test is quite reliable, as the urine very rarely contains substances which can influence the reaction.

3. ***Potassium Iodide and Organic Preparations of Iodine*** (iodol, iodoform, etc.).—Ten to fifteen cc of urine are treated with 5 to 10 drops of yellow nitric acid. One to two cc of chloroform are added, the test-tube closed with a cork, and repeatedly inverted. The chloroform turns a beautiful violet-red from the liberated iodine. The coloration disappears upon the addition of a small quantity of sodium thiosulphate. As has already been mentioned, iodoform separates out during the indican test, and causes a violet-red coloration of the chloroform. This test detects with certainty small quantities of iodine in the urine (0.005).

4. ***Potassium bromide*** and preparations of bromine are also detected by the indican test. The test is not delicate (less than 0.1 cannot be detected).

5. ***Chrysophanic acid*** (dioxymethylanthrachinon) appears in the urine following the use of rheum, senna, chrysarobin, and cascara sagrada. The urine has an intense yellow or greenish-yellow color. Alkaline urine is red. Upon the addition of alkalies, the acid yellow or greenish-yellow urine also becomes red. The red color disappears upon the addition of acetic acid (in contradistinction to blood-pigment). With the indican test, chloroform assumes a greenish coloration.

Urine containing chrysophanic acid strongly reduces *Nylander's* reagent.

6. ***Salicylic Acid and its Preparations*** (salol, salipyrin, salophen, etc.).—The salicylates appear in the urine as salicyluric acid, mono-ethyl-sulphuric esters, combined with glycuronic acid, and partially unaltered, and can be easily detected a very short time following their ingestion. The urine has usually a dark color, which deepens on standing.

For the detection of salicylic acid preparations the urine is treated with 5 to 10 drops of ferric chloride. The solution becomes intensely blue-violet in color; in the presence of smaller quantities it becomes dark red. Since other adventitious constituents of the urine (antipyrin, phenacetin) give a similar reaction, *Marcuse* recommends the following procedure for the identification of salicylic acid: The urine treated as above is further treated with hydrochloric acid, a drop at a time, until a red color is just distinctly present (upon the further addition, the color disappears completely, owing to the decomposition of the ferric salicylate). The solution is then shaken with acetic ether, whereupon the red coloration disappears, if due to salicylic acid; if due to derivatives of antipyrin or phenacetin, the solution is not decolorized.

7. **Antipyrin.**—Following the use of large quantities of antipyrin, the urine is colored yellow to blood-red, and shows a greenish-red fluorescence.

Detection.—(*a*) A dark red color is produced by ferric chloride, which does not disappear upon boiling nor upon shaking with acetic ether.

(*b*) If the urine is treated with a drop of acetic acid and Lugol's iodine potassium iodide solution, a ruby-red crystalline precipitate is formed (*Marcuse*).

8. **Phenacetin** is excreted in the urine partly as phenetidin, partly as para-amidophenol, and partly in coupled combination with glycuronic acid.

Detection.—(*a*) The urine is treated with 2 drops of hydrochloric acid and 2 drops of a 1 per cent. solution of sodium nitrate. If an alkaline watery solution of α-naphthol and a little sodium hydrate are added, a red coloration appears, which, upon the addition of hydrochloric acid, turns violet.

(*b*) With ferric chloride the urine becomes brownish-red.

9. **Balsam of Copaiba.**—(*a*) Treated with hydrochloric acid the urine assumes a pinkish-red color, which on boiling turns red-violet.

(*b*) In performing the albumin test a heavy clouding is produced, which disappears upon the addition of alcohol or petroleum-ether.

10. **Urotropin** enters the urine quickly, and may be detected in it even within half an hour after its ingestion by means of a saturated solution of bromine in water, in which it produces a yellow precipitate, soluble in an excess of urine. It has not yet been proved that urotropin liberates formaldehyde in the urine. The latter is detected in the urine by means of phloroglucin and sodium hydrate. A red color is produced.

11. **Phenol** (carbolic acid) is excreted in the urine as phenol sulphuric acid. The urine is greenish-brown in color, and becomes darker on standing. The dark color of carbolic acid urine depends, according to *Baumann*, upon the formation of hydrochinon. The latter, upon further oxidation, forms brown substances (not more definitely known).

Detection.—Phenol cannot be detected directly in the urine (since the latter contains no free phenol), but must first be isolated. As, however, normal urine contains a small quantity of phenol compounds (about 0.03 in twenty-four hours), only a marked increase is indicative of carbolic acid poisoning. To isolate phenol, a large quantity of urine is distilled after the addition of sulphuric acid (about 5 to 10 cc of sulphuric acid to 100 cc of urine) until the phenol, liberated from the sulphuric acid esters, is all distilled.[1] The distillate is neutralized with pure sodium carbonate and redistilled. This distillate gives the following reactions:

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[1] This is recognized by the fact that the distillate no longer clouds, or produces a precipitate in bromine-water.

(*a*) Upon the addition of a few drops of a neutral solution of ferric chloride a blue coloration appears.

(*b*) With bromine-water a yellowish-white precipitate of tribromphenolbrom is formed. The precipitate is dissolved by sodium hydrate and is reprecipitated from the alkaline solution by hydrochloric acid as tribromphenol, forming yellow crystalline needles.

(*c*) With nitrous acid nitrogen is liberated.

V. Quantitative Chemical Examination of the Urine

1. Estimation of Albumin

(*a*) ***Method of Roberts and Stolnikoff,*** modified by *Brandberg.*

Principle.—If the urine contains 0.0033 gramme of albumin in 100 cc—i.e., 0.033 per litre—the annular clouding with *Heller's* test appears only after two to three minutes. The method depends upon this fact. The urine to be examined is diluted with water until with *Heller's* test a ring is formed only after two to three minutes. From the dilution necessary the albumin contained in the undiluted urine can easily be calculated.

Procedure.—First a dilution of 1:10 is made with the urine to be examined. Five cc of urine are measured with a pipette, put in a glass cylinder, and 45 cc of water added. The mixture is thoroughly shaken and a portion used for *Heller's* test. If after two to three minutes no ring is formed, the dilution is too great, and a dilution of 1: 5 or 1: 3 is made from the undiluted urine. If, however, with the dilution of 1:10 the ring appears at once, the urine must be still further diluted by making dilutions of 1: 30, 50, or 100 from the 1:10 dilution, until a dilution is obtained with which a ring appears only after two to three minutes. With a little practice in carrying out this method, it is easy to approximately estimate the necessary dilution from the intensity of the first clouding, and, therefore the entire estimation may be comparatively quickly made. The quantity of albumin per litre is calculated by multiplying the number 0.033 by the number of the dilution. For example: If the ring appears only after two to three minutes with a dilution of 1: 30 the undiluted urine contains $0.033 \times 30 = 0.99$ gramme of albumin per litre. This method yields results which are sufficiently accurate for clinical purposes. It must, however, be carefully and accurately carried out. It is especially important that *Heller's* test be each time carried out *lege artis.*

(*b*) **Essbach's Method.**

Principle.—Essbach's reagent is composed of a solution of 10 grammes of picric acid and 20 grammes of citric acid in a litre of water. The albumin from a definite amount of urine is precipitated with this reagent, and from the height of the precipitated albumin the amount of albumin contained in the urine is calculated, according to an empirical scale.

Procedure.—Essbach's albuminometer (a graduated tube) is filled with the filtered urine to mark U. The reagent is then added to mark R, the tube closed with a rubber stopper, and, without shaking, inverted ten to twelve times. The tube is stood vertically in a standard, and after twenty-four hours the height of the precipitate is read. The numbers show in grammes the amount of albumin contained in 1,000 cc of urine. The urine must not contain more than 4 per cent. of albumin; when it contains more albumin it must be correspondingly diluted. The method does not give accurate nor reliable results. A precipitate is frequently formed in urine containing no albumin.

(*c*) ***Gravimetric Analysis.***—One hundred cc of filtered urine are treated with 1 to 2 drops of acetic acid, and heated on a water-bath until the albumin has precipitated in flakes. The precipitate is collected upon a filter, which has been previously dried at 110° C., and weighed, washed with water, and then with alcohol and ether, dried at 110° C., and weighed. The filter and its contents are then burned in a platinum crucible, reduced to ashes, weighed, and the weight of the ashes subtracted from the weight of the albumin.

Instead of gravimetric analysis the coagulated and washed albumin can be treated according to *Kjeldahl* (cf. p. 187), and its nitrogen estimated. The amount of albumin is calculated by multiplying the amount of nitrogen obtained by 6.25.

This method yields the only accurate result, but is, unfortunately, too complicated, and consumes too much time for clinical and practical use.

2. Estimation of Sugar

(*a*) ***By Polarization.***—For the quantitative estimation of sugar a *Laurent's* apparatus is best used, which is arranged for white lamplight, and allows the direct reading of the percentage of sugar contained. The urine must be especially prepared for polarization—i.e., the following requirements must be complied with:

1. The urine must be absolutely clear, and must not be deeply colored. Turbid urine must, therefore, be filtered, while highly colored urine must first be decolorized with lead acetate; a few knife-points of powdered neutral

lead acetate are added to about 50 cc of urine, the urine thoroughly shaken and filtered through a dry filter.

2. The urine must be free from albumin, since albumin is lævorotary. When but a slight amount is present (under 0.1 per cent.), this lævorotation may be ignored; when more is present, the albumin must be removed by boiling and the urine brought up to its original volume.

The clear, as nearly as possible colorless, urine is poured into the observation tube of the polarimeter, care being taken that it forms a convexity above the end of the tube; the cover-glass, which must be absolutely clean and dry, is slipped on from the side, so that no air is included; the brass cap is then adjusted. The apparatus is placed at the zero-point, and the observation-tube in it. If the urine contains sugar, the right half of the field will be darkened. The apparatus is adjusted by turning the screw so that both halves of the field are equally bright. The scale then shows the percentage of sugar present.

Polarization is a sufficiently accurate method for practical use. Large quantities of glycuronic compounds, β-oxybutyric acid, and levulose can, however, cause error.

(b) Fermentation Test According to Roberts.

Principle.—The amount of sugar is estimated from the difference in the specific gravity of the urine before and after fermentation. *Roberts* determined by investigation that a reduction of the specific gravity of 0.001 represents 0.230 per cent. of sugar.

Procedure.—The specific gravity at 15° C. is determined, and 100 to 200 cc of urine are fermented in a flask with yeast (a piece the size of a hazel-nut). After twenty-four to thirty-six hours the urine is tested (by the ordinary qualitative tests) to see if the sugar has entirely disappeared. If this is the case, the specific gravity at 15° C. is again determined. Example:

Specific gravity before fermentation . .	1.030
Specific gravity after fermentation . .	1.020
Difference . .	0.010

The urine contains $0.230 \times 10 = 2.30$ per cent. of sugar.

The method gives comparatively accurate results when the determination of the specific gravity is very carefully carried out, and the urine contains not less than 0.5 per cent. of sugar.

(*c*) ***Fermentation Test According to Lohnstein.***—*Lohnstein's* saccharometer consists of a U-shaped tube, the short arm of which has a bulbous enlargement, and can be hermetically closed with a stopper, which has a perforation corresponding to a second in the neck of the bulb. A scale, a *Pravaz* syringe, a weight, a special grease, and a bottle of mercury are supplied with the apparatus.

Sugar is estimated in the following manner: The mercury is poured into the apparatus while both ends of the tube are open; 0.5 cc of the urine to be examined are placed, with the *Pravaz* syringe, upon the surface of the mercury within the bulb. The syringe is then washed with ordinary water, and 1° to 2° (of the scale on the syringe) of yeast paste (prepared with a piece of compressed yeast and a few drops of water) are added. The stopper is greased and placed in the neck of the bulb, so that its perforation corresponds to that in the neck of the bulb. The scale is now set upon the tube of the apparatus. If the meniscus of the mercury is not exactly at the level of the zero line, it is placed there by tipping the apparatus, and the stopper is then turned so that the holes do not correspond with one another. The weight is placed on the stopper, and the urine left to ferment in the apparatus, either at room-temperature or in an incubator. At a temperature of 32° to 38° C. fermentation is completed at the end of three to four hours, even when considerable sugar is present; at ordinary room-temperature it takes six to eight hours. On the removable frame are two scales, one of which is for 20° C., and the other for 35° C. In the majority of cases the 20° scale can be used without marked error. A more exact estimation can be made by means of the following formula:

$$p = p_{35} + \frac{p_{20} - p_{35}}{15} (35 - T).$$

In which p_{20} and p_{35} are the readings on the two scales, and T is the temperature at which the apparatus was filled, and to which it has been again cooled at the completion of the fermentation.

After the estimation the apparatus must be cleansed. The stopper is first turned so that the perforations correspond, then removed, the mixture of urine and yeast swabbed out with a bit of cotton, and the bulb washed out with a stream of water until the water runs away clear. The rest of the water is removed with absorbent cotton. *Lohnstein's* apparatus is to be highly recommended for clinical and practical use. It is especially serviceable in cases in which the percentage of sugar is very small, since it allows accurate readings of 0.1 per cent., or even 0.05 per cent.

(*d*) ***By titration*** after *Pavy-Sahli*.—Solutions required:

SOLUTION NO. 1

Cupri sulfurici crystallisati puri . .	4.158
Aquæ destillatæ ad.	500.0

SOLUTION NO. 2

Salis Seignetti	20.4
Kali caustici puri	25.0
Ammonii caustici (sp. gravity, 0.88) .	300.0
Aquæ destillatæ ad.	500.0

Method of Determination.—5 cc of each of these solutions with 30 cc water are put into an *Erlenmeyer* flask of 75 to 100 cc. The flask is put over a *Bunsen* burner upon an asbestos wire netting and we wait until the contents in the flask begins to boil. In order better to regulate the flame, a piece of copper wire netting is placed over the opening of the *Bunsen* burner. While the solution is being heated, the urine to be examined is diluted ten times (5 cc urine + 45 cc of water) and this diluted solution is put into a burette. As soon as the solution in the flask begins to boil, the flame is reduced somewhat, so that it may continue to boil slowly. The burette is taken into the left hand and the urine solution is allowed to flow into the boiling flask from the burette, drop by drop, care being taken that the boiling be not interrupted until the blue solution of the flask is entirely decolorized. We note the number of cubic centimetres of the urine solution used. The determination is to be considered as at an end only then, when the number of cubic centimetres used up is between 5 and 10. If less than 5 cc have been used a second determination must be made whereby a greater dilution of the urine is made.[1] The quantity of the urine dilution used up at the first titration guides us in making the second dilution. If at the first titration less than 1 cc was used up, we prepare another solution of the urine which has been diluted one hundred times.

If 1 to 1.5 cc were used then a dilution of 50
" 1.5 to 2.5 " " " " 40
" 2.5 to 5.0 " " " " 20

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[1] The second dilution is absolutely necessary, as accurate results can be obtained only if the urine contains 0.5 to 1.0 sugar per mille.

The sugar determination is made in the following manner. 0.005 gr. of grape-sugar is required to reduce 10 cc of *Pavy's* solution used. Therefore the used up quantity of the urine solution contains 0.005 glycose. If at the second titration 8 cc were used of the urine which was diluted 20 times, then such urine contains

$$\frac{0.005 \;.\; 20 \;.\; 100}{8} = 1.25 \text{ per cent. sugar.}$$

The examination lasts ten to twenty minutes.

3. Estimation of Total Nitrogen

The nitrogen of the urine is usually estimated according to *Kjeldahl's* method.

Principle.—The various nitrogenous substances are converted into ammonium sulphate by boiling with sulphuric acid. The ammonia is freed from the ammonium sulphate by supersaturation with a solution of sodium hydrate, and collected in a titrated solution of sulphuric acid. From the amount of acid bound by the ammonia, the NH_3 contained is calculated, and from it the N is calculated.

Procedure.—Five cc of urine are treated in a so-called *Kjeldahl*-flask (of hard glass) with 10 cc of *Kjeldahl*-sulphuric acid (a mixture of three parts pure and one part fuming sulphuric acid) and a few drops of a saturated solution of copper sulphate. The flask is then heated on a sand-bath in a fume-chamber until the solution is decolorized. The solution is then allowed to cool, and, with agitation, 50 cc of distilled water are added. The solution, which has again become hot, is poured into a litre distilling-flask, and the *Kjeldahl*-flask rinsed two or three times with water, which is also poured into the distilling-flask. The solution is now rendered alkaline with

40 per cent. sodium hydrate (about 40 to 60 cc)[1] and distilled at once. The addition of the sodium hydrate must be made quickly to avoid loss of ammonia. The distilling-tube must have a bulb to prevent the sodium hydrate from being carried over into the receiver. The distillate is collected in a receiver containing 50 cc of decinormal sulphuric acid. The distillation is completed twenty to thirty minutes after boiling has begun, which is indicated by marked bumping (due to beginning precipitation of sodium sulphate). The stopper is then removed from the distilling-flask, the flame extinguished, the distilling-tube washed with water into the receiver, and the contents of the latter titrated with decinormal sodium hydrate, using rosolic acid as an indicator. The alkali is added until a permanent pink coloration of the solution is produced. The number of cc of decinormal alkali used is subtracted from the number of cc of decinormal sulphuric acid used in the receiver. The difference multiplied by 0.0014 gives the number of grammes of nitrogen contained in the quantity of urine used, from which the percentage is calculated. It is advisable, as a control, to carry out simultaneously two tests with samples of the same urine.

4. The Determination of Urates

(*a*) *Pflueger-Bleibtreu's Method.*

Principle.—This method depends on the principle that with the exception of the urates all other nitrogen compounds of the urine are precipitated by phospho-tungstic acid. The urates are then removed by phosphoric acid and the nitrogen contained in them is determined after *Kjeldahl.*

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[1] If the sp. gr. of the urine is higher than 1020, 75 cc. of $\frac{1}{10}$ normal sulphuric acid is added.

The necessary reagents are:

1. A solution of phospho-tungstic acid (9 parts of a 10 per cent. phospho-tungstic acid plus one part hydrochloric acid of the sp. gr. 124).
2. Phosphoric acid crystals.
3. Powdered calcium hydrate.
4. The reagents necessary for a *Kjeldahl* determination.

Determination: Having previously determined the absolute quantity of phospho-tungstic acid required for the absolute precipitation, such quantity of the acid is added to 50 cc of the urine into a flask of 200 cc. This is diluted up to 150 cc with a 10 per cent. solution of HCl; filtration after twenty-four hours; the clear filtrate is rubbed up with the calcium hydrate powder until the reaction is alkaline; again filtration; 15 cc (corresponding to 5 cc of urine) to which 10 gr. of the crystalline phosphoric acid was added, are put into a flask, which is then left in a drying oven, kept at a temperature of 150°, for four and one half hours and the day substance, left after the evaporation, is dissolved in warm water and the nitrogen is determined after *Kjeldahl.* The quantity of nitrogen obtained is multiplied by $\frac{15}{7} = 2\frac{1}{7}$. In this way are determined the urates of 5 cc of urine. There is a difference of opinion among authorities, whether ammonia is precipitated by the phospho-tungstic acid, or whether it passes into the filtrate together with the urates.

Gumlich holds that all the ammonia is precipitated by *Merck's* preparation of the phospho-tungstic acid. Whilst *Pflueger* and *Bleibtreu* maintain that the ammonia is determined together with the urates by the method described. They therefore recommend, that the ammonia be determined after the method of *Schloesing* and that the quantity of nitrogen thus determined be deducted from what has been previously obtained by phospho-tungstic acid. The nitrogen of the urates equals the difference of these two determinations.

(*b*) ***Knop and Huefner's Method.***

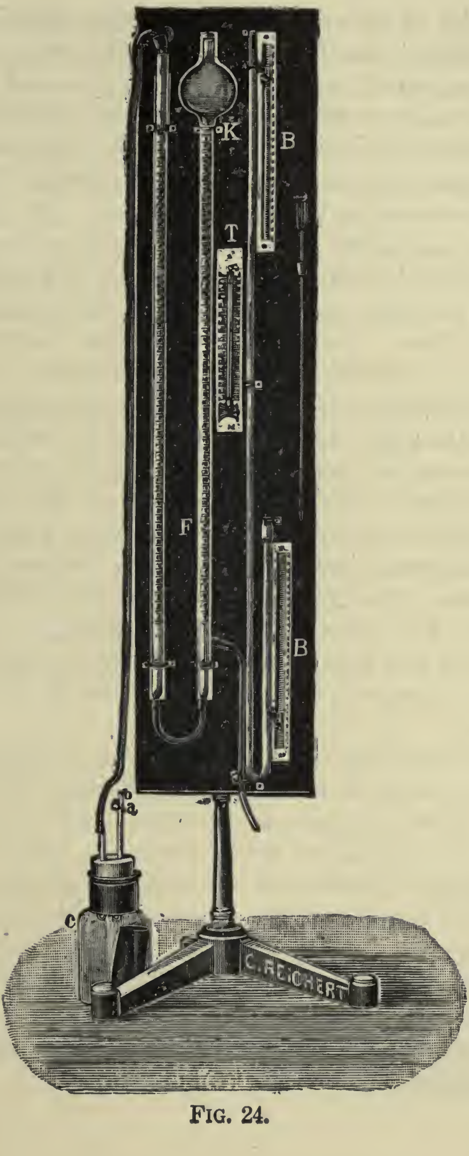
Principle.—The urea is decomposed with an alkaline solution of sodium hypobromite into nitrogen, carbon dioxide, and water.

$$CO < \begin{matrix} NH_2 \\ NH_2 \end{matrix} + 3BrONa = CO_2 + 2H_2O + 2N + 3NaBr.$$

The carbon dioxide is bound by the soda, while the quantity of nitrogen liberated is estimated volumetrically. From this the urea is calculated.

Procedure.—The estimation is best carried out with the apparatus recently constructed by *Jolles*. *Jolles'* azotometer (Fig. 24) consists of a mixing-jar and two graduated tubes. These three parts of the apparatus are joined by rubber tubing. The mixing-jar (*c*) contains a smaller cylindrical jar of hard rubber or glass. In addition to the rubber tube connecting the mixing-jar with the graduated tubes, a second tube with a free end passes through the rubber stopper of the jar. This tube is closed at its outer end with a pinch-cock. The urea is estimated in the following manner:

Ten cc of the filtered urine are treated in a 100 cc graduated flask with about 30 cc of distilled water, and sufficient phospho-tungstic acid containing hydrochloric acid for precipitation (100 cc of HCl of a specific gravity of 1.124 + 900 cc of 10 per cent. phospho-tungstic acid). The flask is heated, with agitation, in a water-bath for a quarter of an hour, allowed to stand four hours, filled to the mark with distilled water, shaken, and the contents filtered through a dry filter. Twenty-five cc of the filtrate (= 2.5 urine) are withdrawn with a pipette, placed in the


K
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C. REICHERT

FIG. 24.

mixing-jar of the azotometer, and, with agitation, carefully rendered alkaline. Thirty cc of bromine solution (100 grammes of sodium hydrate are dissolved in 250 cc of water, and 25 grammes of bromine are added to the cold solution) are placed in the smaller inner vessel. The inner vessel is best held in a pair of forceps when introduced, to avoid mixing the two fluids. The graduated tubes are now filled with ordinary water to the mark O, with the pinch-cock on the tube of the mixing-jar open, in such a manner that the liquid in the two tubes is at the same level. The pinch-cock is then closed and the mixing-jar inclined, so that the two fluids mix, whereupon a lively formation of gas takes place. The water which rises in the right-hand tube is allowed to escape through the outlet, which is near the bottom of the tube, until its level is about 1 to 2 centimetres higher than that of the water in the other tube. The jar is shaken a few minutes, care being taken that no liquid enters the free end of the glass tube. After gas formation has ceased at least fifteen minutes, the water in the tubes is brought to the same level, and the level noted. From the number of cc of nitrogen developed, the urea is calculated in the following manner:

The temperature and the height of the barometer are estimated,[1] and the number of cc of N found is multiplied by the coefficient in the table on pp. 194, 195, corresponding to the temperature and pressure.

Example.—18.6 cc of nitrogen, temperature 16° C., height of barometer 760, coefficient in the table 0.998. The amount of urea contained is $0.998 \times 18.6 = 18.56$ grammes per litre. Thus the table renders possible a direct estimation of the amount of urea per litre. For rapid practical use the estimation of urea with the azotometer can be much simplified by omitting the precipitation with phospho-tungstic acid. The entire estimation then takes but fifteen to twenty minutes; 2.5 cc of urine are measured with an accurate pipette and placed in the small jar. Thirty cc of the bromine solution and 100 cc of distilled water are placed in the mixing-jar, and the nitrogen estimated in the above-described manner. The urea is calculated from the table on pp. 194 and 195.

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[1] As seen in Fig. 24, there are added to the apparatus a thermometer (T) and a barometer (B).

5. Estimation of Uric Acid

(*a*) ***Hopkins' Method.***—One hundred cc of urine are saturated with 25 grammes of sodium chloride, and set aside for twenty-four hours. The precipitate is then collected on a filter and washed free from chlorine with a 10 per cent. solution of ammonium sulphate—i.e., until the filtrate no longer becomes clouded upon the addition of a solution of silver nitrate. The precipitate is then washed, without loss, with hot water into an *Erlenmeyer* flask, and the solution allowed to cool. Twenty cc of concentrated sulphuric acid are now added, and the solution, which has again become hot, is at once titrated with a $\frac{1}{20}$ normal permanganate solution. The permanganate solution is added until the red coloration, which is produced, lasts a few seconds. Each cc of the permanganate solution represents 0.00361 gramme of uric acid.

This method gives comparatively accurate and useful results.

(*b*) ***Kowarsky's Simplified Method.***

Exactly 10 cc of urine are measured off with a pipette and put into a thin walled centrifuge tube of about 15 cc. Two to three drops ammonia and 3 gr. of powdered ammonium chlorid are added. (The ammonium chlorid

TABLE FOR THE

1 cc of nitrogen represents grammes of

Height of Barometer.	10°	12°	14°	15°	16°
700.........	0.934	0.943	0.926	0.922	0.917
702.........	0.945	0.937	0.929	0.925	0.920
704.........	0.948	0.940	0.932	0.927	0.923
706.........	0.951	0.943	0.934	0.930	0.926
708.........	0.954	0.945	0.937	0.932	0.928
710.........	0.957	0.948	0.939	0.935	0.931
712.........	0.959	0.951	0.942	0.938	0.933
714.........	0.962	0.953	0.945	0.941	0.936
716.........	0.964	0.956	0.948	0.944	0.938
718.........	0.967	0.959	0.951	0.946	0.941
720.........	0.970	0.962	0.953	0.949	0.944
722.........	0.973	0.964	0.956	0.951	0.947
724.........	0.975	0.967	0.958	0.954	0.950
726.........	0.978	0.970	0.961	0.957	0.952
728.........	0.981	0.973	0.964	0.959	0.955
730.........	0.984	0.975	0.967	0.962	0.957
732.........	0.987	0.978	0.969	0.965	0.960
734.........	0.989	0.981	0.972	0.968	0.963
736.........	0.992	0.983	0.975	0.970	0.966
738.........	0.995	0.986	0.977	0.973	0.969
740.........	0.998	0.989	0.980	0.975	0.971
742.........	1.000	0.992	0.982	0.978	0.974
744.........	1.003	0.994	0.985	0.981	0.976
746.........	1.005	0.997	0.988	0.983	0.979
748.........	1.008	0.999	0.991	0.986	0.981
750.........	1.011	1.002	0.993	0.989	0.984
752.........	1.014	1.005	0.996	0.992	0.987
754.........	1.017	1.008	0.999	0.994	0.990
756.........	1.019	1.011	1.001	0.997	0.992
758.........	1.022	1.013	1.004	0.999	0.995
760.........	1.025	1.016	1.007	1.002	0.998
762.........	1.028	1.018	1.010	1.005	1.000
764.........	1.030	1.021	1.012	1.008	1.003
766.........	1.033	1.024	1.015	1.011	1.006
768.........	1.036	1.027	1.017	1.013	1.008
770.........	1.039	1.029	1.020	1.016	1.011

ESTIMATION OF UREA.

urea per litre ; temperature in Centigrade.

17°	18°	19°	20°	21°	23°	25°
0.913	0.909	0.904	0.900	0.895	0.886	0.877
0.916	0.911	0.907	0.903	0.898	0.889	0.879
0.919	0.914	0.909	0.905	0.901	0.891	0.882
0.921	0.917	0.912	0.908	0.903	0.894	0.885
0.924	0.920	0.915	0.910	0.906	0.897	0.887
0.927	0.922	0.917	0.913	0.909	0.899	0.890
0.929	0.925	0.920	0.916	0.911	0.902	0.892
0.932	0.927	0.923	0.919	0.914	0.904	0.895
0.934	0.930	0.926	0.921	0.916	0.907	0.897
0.937	0.933	0.928	0.924	0.919	0.910	0.900
0.940	0.935	0.931	0.927	0.921	0.912	0.903
0.943	0.938	0.933	0.929	0.924	0.915	0.905
0.945	0.940	0.936	0.932	0.927	0.917	0.908
0.948	0.943	0.938	0.934	0.930	0.920	0.910
0.951	0.946	0.941	0.937	0.933	0.922	0.913
0.954	0.949	0.944	0.939	0.935	0.925	0.915
0.956	0.951	0.947	0.942	0.938	0.928	0.918
0.959	0.954	0.950	0.945	0.940	0.931	0.921
0.961	0.957	0.952	0.947	0.943	0.933	0.923
0.964	0.959	0.955	0.950	0.945	0.936	0.926
0.967	0.962	0.957	0.952	0.948	0.938	0.928
0.969	0.964	0.960	0.955	0.951	0.941	0.931
0.972	0.967	0.962	0.958	0.953	0.944	0.934
0.975	0.970	0.965	0.961	0.956	0.946	0.937
0.977	0.973	0.968	0.963	0.958	0.949	0.939
0.980	0.975	0.970	0.966	0.961	0.951	0.942
0.982	0.978	0.973	0.968	0.963	0.954	0.945
0.985	0.981	0.975	0.971	0.966	0.957	0.947
0.988	0.983	0.978	0.974	0.969	0.959	0.950
0.991	0.986	0.981	0.976	0.971	0.962	0.952
0.993	0.988	0.984	0.979	0.974	0.964	0.955
0.996	0.991	0.987	0.981	0.976	0.967	0.957
0.999	0.993	0.989	0.984	0.979	0.969	0.960
1.001	0.996	0.992	0.987	0.981	0.972	0.963
1.004	0.999	0.994	0.989	0.984	0.974	0.965
1.006	1.002	0.997	0.992	0.987	0.977	0.968

powder may be ordered in doses of 3 gr. from the druggist.) The tube is closed up with a rubber stopper and thoroughly shaken up until all of the ammonium chloride is dissolved. Ammonium urate is separated in the form of a flocculent sediment. The phosphates, which are likewise sedimented, do not interfere with the determination. The tube is left for two hours so that all the ammonium muriate may separate. The tube is then centrifugalized for one to two minutes which causes all of the sediment to settle at the bottom of the tube; the clear fluid is then poured off, usually without any loss of the sediment. While pouring off the fluid the tube should be inclined but once, as by repeated inclinations the sediment is disturbed, whereby some of it may be lost.

The sediment, to which five drops of concentrated HCl is added, is carefully heated over a small flame; the ammonium muriate is thereby dissolved and the separation of the free uric acid begins at once in the form of a crystalline sediment. The tube is left alone for one hour for the thorough separation of the uric acid. The separated uric acid is shaken up, about 2 cc of water are added and then centrifugalized; about ten revolutions are sufficient to throw down the crystalline sediment to the bottom; the fluid is now poured off, the sediment again shaken up with 2 to 3 cc of alcohol and again centrifugalized. Two or three more times the sediment is washed with alcohol until the alcohol reacts neutral to litmus. The washing of the sediment lasts mostly from three to five minutes. After the last alcohol was poured off a few cc of water are heated in a test-tube; about two cc of the hot water is poured over the again shaken up sediment, one drop of phenol-phthalein is added and the hot solution is titrated with a $\frac{1}{50}$ normal solution of piperidin. This latter solution is added to the hot solution so long (the hot solution being shaken all the time while the titration lasts) until this hot solution remains of a pink color permanently, even after it is heated up again.

To get the number of *mg* of uric acid contained in 10 cc of urine, we multiply the number of cc of the piperidin solution used up in the titration by 3.36. If, for instance, 1.5 cc of the piperidin solution have been used up in the titration, we find the number of mg of uric acid contained in 10 cc of urine by multiplying $3.36 \times 1.5 = 5.04$ mg. Therefore in 100 cc $5.04 \times 10 = 50.4$ mg or 0.0504 g., i.e., 0.0504 per cent. The piperidin solution can be kept for some time and its usability can be tested with a $\frac{1}{50}$ normal HCl or sulphuric acid solution.

(*c*) ***Salkowski and Ludwig's Method.***

Principle.—The uric acid is precipitated as a salt of silver and magnesium, and the uric acid liberated from the silver precipitate is determined gravimetrically, or calculated from the nitrogen estimated according to *Kjeldahl.*

NECESSARY SOLUTIONS.—1. *An Ammoniacal Solution of Silver.*—Twenty-six grammes of silver nitrate are dissolved in water in a litre flask, ammonia is added until the precipitate formed is redissolved, and water added to the mark.

2. *Magnesium Mixture.*—One hundred grammes of crystalline magnesium chloride are dissolved in water in a litre flask, ammonia added until the mixture smells strongly of it, and then a cold saturated solution of ammonium chloride added, until the precipitate of magnesium hydrate which is formed is redissolved; the flask is then filled to the mark with water.

3. *A Solution of Potassium* (*or Sodium*) *Sulphate.*—Fifteen grammes of potassium hydrate or 10 grammes of

sodium hydrate are dissolved in a litre of water; half the solution is saturated with hydrogen sulphide, and then mixed with the other half.

Procedure.—Ten cc of magnesium mixture and 10 cc of the silver solution are mixed in a beaker, and treated with ammonia until the precipitate of silver chloride is redissolved. One hundred cc of urine are added to the mixture, with stirring. The precipitate containing uric acid which is formed at once is collected on a filter, but it is not necessary to remove the precipitate which has collected on the sides and bottom of the beaker. The filter and the precipitate are placed in the same beaker in which the precipitation was carried out. Ten cc of the potassium sulphate solution and 10 cc of water are added and heated to the boiling-point (prolonged and vigorous heating is to be avoided, as the uric acid can be oxidized by it). The hot solution is filtered, and the residue washed with hot water. The filtrate is collected in a porcelain dish, and the sodium urate contained in it decomposed by the addition of a small amount of hydrochloric acid. After it has evaporated down to about 15 cc, and a few more drops of hydrochloric acid have been added, it is set aside for twelve to twenty-four hours. The precipitated free uric acid is collected on a small weighed filter, washed with water, ether, alcohol, and carbon bisulphide, dried, and weighed. The amount of the crystallized uric acid can be more simply determined by the estimation, according to *Kjeldahl*, of the nitrogen contained. The uric acid collected on the filter is then washed with a small quantity of water, the filter and precipitate placed in a *Kjeldahl*-flask, and the further estimation carried out as in estimating nitrogen in the urine. The quantity of nitrogen contained is multiplied by 3. This method is more complicated, and consumes more time than that of *Hopkins*, but it yields more accurate and useful results.

6. Estimation of Chlorides

According to Mohr.

Principle.—If a solution of sodium chloride is treated with a little potassium chromate, and then a solution of silver, silver chloride is precipitated. After all the chlorine has combined with silver, further addition of the silver solution produces silver chromate, which colors the precipitate orange.

NECESSARY SOLUTIONS.—1. *Silver Solution.*—This is made by dissolving 29.042 grammes of pure silver nitrate in a litre of distilled water.

2. *A* 10 *per cent. solution of potassium chromate.*

Procedure.—Ten cc of urine are diluted in a flask or beaker with 30 to 50 cc of distilled water, and treated with a few drops of the potassium chromate solution, until a distinct yellow coloration is produced.

The silver solution is then run in from a burette, with vigorous agitation, until the reddish coloration no longer disappears as at first. One cc of the silver solution represents 0.01 gramme of sodium chloride.

This method gives results sufficiently accurate for clinical and practical use; more accurate results are obtained if the urine is reduced to ashes, and the chlorine in the ashes estimated according to the same method.

7. Estimation of Phosphates

Volumetric Analysis.

Principle.—If phosphates in a hot acetic acid solution are brought in contact with a solution of uranium nitrate, the phosphoric acid is entirely precipitated as uranium phosphate.

NECESSARY SOLUTIONS.—1. *A Solution of Sodium Acetate in Acetic Acid.*—One hundred grammes of sodium acetate are dissolved in 800 cc of water, 100 cc of 30 per cent. acetic acid added, and the solution brought up to a litre.

2. *Uranium Nitrate Solution.*—This solution contains about 14 grammes of uranium nitrate to a litre of water, and is made with an accurately prepared solution of sodium phosphate, which contains 0.1 P_2O_5 in 50 cc. One cc of this solution represents 0.005 gramme of phosphoric acid.

3. *A* 10 *per cent. solution of potassium ferrocyanide.*

Procedure.—Fifty cc of urine in an *Erlenmeyer* flask are treated with 5 cc of the acetic acid sodium acetate solution, and heated to the boiling-point. The uranium nitrate solution is now run in as long as the formation of a precipitate can be distinctly seen; a drop of the liquid is then tested with potassium ferrocyanide after the addition of each ½ cc to determine if the end-reaction has taken place. For this purpose a series of drops of the potassium ferrocyanide solution are placed on a porcelain dish, and a drop removed from the solution with a glass rod is allowed to run into one of them. If a reddish-brown coloration appears at the point of contact of the two drops, the end-reaction has taken place (potassium ferrocyanide combines with the uranium, forming uranium ferrocyanide, which forms a reddish-brown precipitate). The number of cc of the uranium solution used, multiplied by 0.005, gives the amount of P_2O_5 in 50 cc of urine. Instead of potassium ferrocyanide, tincture of cochineal may be used as indicator; the hot solution is treated with 1 to 2 cc of the tincture, and titrated with uranium nitrate until it becomes grass-green. The urine must be free from albumin. The method gives good results.

8. Estimation of Sulphates.

Sulphuric acid appears in the urine in two forms, in the form of sulphuric acid salts (=preformed sulphates), and in combination with aromatic alcohols, as phenol, indoxyl, and brenzkatechin (–O–dihydroxylbenzol = $C_6H_4[OH]_2$) as sulphuric acid esters or combined sulphuric acid.

(*a*) *Estimation of the Preformed Sulphates.*

Principle.—The sulphuric acid is precipitated in an acid solution with barium chloride, and estimated gravimetrically.

Procedure.—Fifty to one hundred cc of the filtered urine are diluted with an equal quantity of water, acidified with acetic acid, treated with an excess of barium chloride solution, and heated on a water-bath until the precipitate of barium sulphate has settled. The precipitate is then collected on an ash-free filter, washed with hot water until it is entirely free from chlorine (no clouding upon the addition of silver nitrate and nitric acid), the filter and precipitate then dried, and reduced to ashes in a platinum crucible. After it has cooled, the platinum crucible and the ashes are weighed, the weight of the crucible subtracted, and the difference multiplied by 0.34331. The product represents the SO_3 contained in the urine used.

(*b*) *Estimation of the Combined Sulphuric Acid.*

The filtrate from the above estimation is strongly acidified with hydrochloric acid (by the addition of 10 cc of HCl), boiled for some time, and, if necessary, a few drops of a hot solution of barium chloride are added. The combined sulphuric acid is liberated by the boiling with hydrochloric acid, and precipitated as a barium salt. The precipitate is collected upon an ash-free filter, and the further estimation carried out as in estimating the preformed sulphates.

If the results of both estimations are added, the total sulphuric acid is obtained. This is, in the normal adult, 1.5 to 3 grammes SO_3 in the twenty-four hours' sample of urine.

9. Estimation of Oxalic Acid According to Salkowski

Principle.—The oxalic acid is extracted with alcohol and ether from urine containing hydrochloric acid: after distillation of the alcohol and ether, the oxalic acid is precipitated as a calcium salt, and estimated gravimetrically.

Procedure.—Five hundred cc of unfiltered urine are evaporated down, on a water-bath, to about 150 cc, treated, after cooling, with 20 cc of concentrated hydrochloric acid, and placed in a 500 cc separatory funnel. The mixture is shaken three times with an equal quantity of alcohol and ether (nine parts ether, one part absolute alcohol), and the ether extract is collected in a flask. The ether extract is then filtered through a dry filter into a dry distilling-flask, and the ether distilled. The fluid remaining in the flask is poured into a porcelain dish, and the flask is rinsed first with alcohol, and then with water, pouring these also into the dish. The dish is heated on a water-bath (a little water being added) until the odor of alcohol and ether has disappeared. The watery solution which remains (its volume should be about 20 cc) throws down the resinous substances on cooling. The solution is filtered, the filtrate rendered slightly alkaline with ammonia, treated with 1 to 2 cc of a 10 per cent. solution of calcium chloride, and acidified with acetic acid. The solution is then set aside in a warm place for some time (twelve to twenty-four hours) until the precipitate of calcium oxalate has collected on the bottom of the receptacle. The precipitate is then collected, without loss, on an ash-free filter, washed with water, dried, thoroughly burned (to convert the calcium oxalate into caustic lime), and weighed. The weight of the caustic lime (CaO) multiplied by $\frac{5}{3}$ gives the amount of oxalic acid. If the estimation is properly carried out, the caustic lime gives off no carbon dioxide when dissolved in dilute hydrochloric acid; the solution must also give a negative reaction when tested for phosphoric acid with ammonium molybdate. This method yields accurate results. Normal urine contains not more than 0.02 gramme of oxalic acid in twenty-four hours.

10. Schloesing's Method of Determining Ammonia

Principle.—Ammonia is liberated by milk of lime and taken up by sulphuric acid in a closed vessel.

Solutions Required.—1. One-quarter normal sulphuric acid. 2. One decinormal solution of sodium hydrate.

Determination.—Twenty-five cc of filtered urine are put into a flat dish, the walls of which run up perpendicularly, and this dish is put on the plate of a large exsiccator (this consists of a glass plate and a glass bell cover). A triangle made of glass is put into the dish and on it is put a small dish into which are added 20 cc of a one-quarter normal sulphuric acid by means of a pipette. To the urine are added at least 10 cc of milk of lime and the glass plate is now covered with the glass bell, the border of which has previously received a coat of lard. After three to four days almost all of the ammonia has been expelled and absorbed by the sulphuric acid. If the moisture which settled on the inner wall of the bell reacts alkaline, it is washed into the sulphuric acid. By the titration with the decinormal sodium hydrate solution is determined how much of the ammonia has combined with the sulphuric acid; 1.7 mg ammonia corresponds to 1 cc of the decinormal sulphuric acid solution (20 cc of $\frac{1}{4}$ normal acid corresponds to 50 cc $\frac{1}{10}$ normal acid). In this titration methyl orange is used as an indicator.

11. Messinger's Method of Determining Acetone

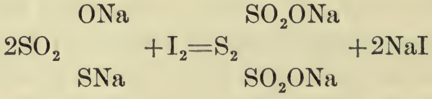
Principle.—In an alkaline solution acetone is changed into iodoform by iodine-kalium iodide. The iodine does not act as such on the acetone, but as an iodide which forms by the reaction of the iodine upon the alkaline hydrate. The reaction takes place as follows:

$$I_2 + KOH = KOI + HI;\ HI + KOH = KI + H_2O$$

$$\underset{\text{Acetone}}{CH_3COCH_3} + \underset{\text{Kalium iodid}}{3KOI} = CH_3COCI_3 + 3KOH$$

$$CH_3COCI_3 + KOH = CH_3COOK + \underset{\text{Iodoform}}{CHI_3}$$

In the transformation of one molecule of acetone into one molecule of iodoform, 6 atoms of iodine are required. First is added iodine in excess, and the remaining quantity of iodine is then determined by titration with natrium-thio-sulphate which is changed into tetra-thio-sodium.



$$2SO_2\begin{matrix}ONa\\ \\SNa\end{matrix} + I_2 = S_2\begin{matrix}SO_2ONa\\ \\SO_2ONa\end{matrix} + 2NaI$$

The quantity of the iodine is calculated from the quantity of the iodine in combination.

Solutions Required.—1. One-tenth normal solution of iodine. 2. One-tenth normal solution of thio-sulphate. 3. Fifty per cent. solution of acetic acid. 4. Sulphuric acid diluted eight times. 5. A starch solution.

Determination.—The acetone is distilled off the urine. The distillate must not contain either phenol, or ammonia, or nitrous acid, or formic acid. One hundred cc of urine are distilled together with 2 cc of a 50 per cent. solution of acetic acid (acetic acid keeps back the phenol). This distillate is again distilled with 1 cc of the sulphuric acid solution (solution No. 4, above), ammonia combining with the sulphuric acid. Each distillation is continued until only one-fourth of the solution remains.

Into a flask, having a good fitting glass stopper, is put the second distillate to which is added in excess the iodine solution (2) (50 to 100 cc); the flask is shaken up, and then is added drop by drop in excess a concentrated solution of sodium hydrate which is free from nitrites. The iodine color disappears and iodoform settles at the bottom. The flask is now stoppered, well shaken up and left for five minutes. The fluid is then acidulated with concentrated hydrochloric acid, whereby the liberated iodine separates. The titration with the thio-sulphate solution is continued until the dark brown color has changed to a faint yellow; now a few cc of the starch solution are added. There appears at first a green or a brownish green color; on the addition of more thio-sulphate a pure blue color results. The titration is continued until the fluid is entirely decolorized; toward the end the thio-sulphate is added drop by drop. Each cc of the iodine solution in combination corresponds to 0.967 mg of acetone.

Illustration.—Assuming 100 cc of the iodine solution have been added and in the titration 23 cc of thio-sulphate have been used. Since 1 cc of the thio-sulphate solution corresponds to 1 cc of the iodine solution, therefore 100 − 23 cc = 77 cc of the iodine solution were used to form the iodoform.

Therefore 1 litre contains 77×0.967 mg=74.459 mg in 100 cc of urine or 0.74459 gr to a litre of acetone.

VI. Examination of Urinary Calculi and Concretions

According to their composition, the following varieties are distinguished:

1. Uric acid stones, which are composed of free uric acid, acid sodium urate, or (more rarely) ammonium urate.
2. Phosphatic stones, which are composed principally of phosphates of calcium, magnesium, and calcium carbonate.
3. Oxalate stones, composed of calcium oxalate.
4. Cystin and xanthin stones (very rare concretions).
5. Mixed stones, which are composed of layers of varying composition.

General Characteristics

Color.—Uric acid stones are yellow to dark brownish-red; phosphatic stones are whitish, grayish to grayish-yellow; oxalate stones usually brownish-red to black, though occasionally they are white or gray (the smaller stones); cystin stones are pale yellow; xanthin stones light brown.

Surface.—Oxalate stones have a rough, irregular, or warty surface (mulberry stones); uric acid stones a less rough; phosphatic stones usually a sandy, comparatively smooth surface; cystin and xanthin stones are usually smooth.

Consistency.—The softest stones are the cystin and phosphatic stones; the latter are more or less earthy or chalky, and comparatively crumbly in consistency. Cystin stones are of waxy softness, uric acid stones much harder, and oxalate stones are the hardest.

Chemical Examination

For examination the stones are sawn into two equal parts, the cut surface smoothed a little, and washed with water. The strata, of which the stone is composed, and the nucleus then appear distinct. For chemical examination, a portion is scraped from each stratum and from the nucleus, and each portion is examined separately. If no stratification and no nucleus are seen on the cut surface, the stone is crushed and rubbed to a fine powder in a mortar. A small portion of the powder is burned on a platinum spatula. This preliminary test determines the course of further chemical examination, since the excess of organic or inorganic matter in the stone is determined by it. Two things may happen:

1. Almost all the material burns up and very little or no residue is left—i.e., the stone is composed principally of organic matter. Such stones may be composed of uric acid, urates, xanthin, or cystin. Uric acid and xanthin stones burn without flame, and with an odor suggesting hydrocyanic acid; cystin stones with a bluish flame, and an odor suggesting sulphurous acid.

To determine which of the above-mentioned organic substances constitutes the main portion of the stone, a second portion is evaporated to dryness in a porcelain dish with a few drops of nitric acid. If the residue turns purple-red on the addition of a drop of ammonia, and blue-violet on the addition of a drop of sodium hydrate (murexide test), it is composed of uric acid, ammonium urate, or some other urate. If the original substance liberates ammonia upon the addition of potassium hydrate, the stone is composed of ammonium urate. If the test for ammonia is negative, and the stone burns up completely, it is composed of pure uric acid. Other urates leave a slight residue on burning.

If with the murexide test no coloration is produced with ammonia, while with sodium hydrate a beautiful red coloration is produced, the stone is composed of xanthin. Cystin stones give a negative result with the murexide test, both with ammonia and sodium hydrate. They are distinguished by the fact that they are readily soluble in ammonia, and that when the ammonia is slowly evaporated, the cystin crystallizes in very characteristic hexagonal plates.

2. The material does not burn at all, or merely turns black, and leaves a very marked residue when burned. In this case, the stone may be principally composed of phosphates, carbonates, or oxalates. A small portion is slightly heated with dilute hydrochloric acid, whereupon the majority of the powder is dissolved. Only the organic basic substance, and any uric acid which may be present, remain undissolved. The solution is allowed to cool (in order to precipitate the uric acid), filtered, the filtrate diluted with water, and rendered strongly alkaline with ammonia. If a precipitate is produced by the addition of ammonia, it may consist of:

(*a*) Earthy phosphates (calcium and magnesium phosphates), triple phosphates (ammonium magnesium phosphate); or,

(*b*) Calcium oxalate.

The precipitate is separated from the solution (best by centrifugalization) and dissolved in acetic acid. The triple phosphates and earthy phosphates are thus dissolved, while calcium oxalate remains undissolved, and can be detected microscopically.

The following test is carried out with the filtered acetic acid solution. It is treated with ammonium molybdate and nitric acid, and heated to 60° C. If a yellow precipitate is formed, phosphoric acid is present.

If upon the addition of ammonia to the hydrochloric acid solution of the stone no precipitate is formed, it may be composed of calcium or magnesium carbonate.

A portion of the stone is then touched with hydrochloric acid, by which gas (CO_2) will be liberated. One-half of the ammoniacal solution is treated with ammonium oxalate; if a precipitate of calcium oxalate is produced, calcium carbonate is present. To the other half a solution of sodium phosphate is added; if a precipitate of triple phosphate is produced, magnesium carbonate is present.

The portion of the stone undissolved by hydrochloric acid must be tested for uric acid with the murexide test.

VII. Microscopical Examination of the Urinary Sediment

There are three methods for collecting urinary sediment for microscopical examination:

1. ***Sedimentation in a Conical Glass.***—The urine is allowed to stand undisturbed for some time in a conical glass; solid insoluble constituents gradually sink and collect as a precipitate at the apex of the glass. After the solution has been decanted as completely as possible, a drop of the sediment is removed with a pipette for examination.

2. ***Collection of the Precipitate on a Filter.***—As large a quantity as possible of the urine to be examined is filtered through a moist filter, upon which the solid constituents collect.

3. ***Precipitation of the Solid Constituents by Centrifugalization of the Urine.***—A small glass tube with a conical bottom is filled with the urine,[1] placed in the holder of a

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[1] It is advisable to allow the urine to stand one to two hours before taking the portion to be examined, and then to remove the lowest portion with a long pipette, and centrifugalize it.

centrifuge, and centrifugalized for a few minutes. The insoluble constituents are thus collected at the apex of the centrifuge-tube. The liquid above the sediment is poured off by inverting the tube as quickly as possible. The liquid should not be poured off gradually, as the sediment is then apt to become mixed with it.

The advantages of centrifugalization are evident. By sedimentation in a conical glass urine containing but few solid constituents yields almost no precipitate, while when centrifugalized it yields sufficient sediment for examination. Moreover, with the latter method it is not necessary for the urine to stand for hours, during which decomposition, and therefore alteration of its solid constituents, easily take place. A drop of the sediment is removed with a pipette, placed on a slide, and, without pressure, is covered with a cover-glass. If the solution extends beyond the cover-glass, the excess must not be absorbed with filter-paper, since by so doing the solid constituents might be drawn out from under the cover-glass with the fluid. It is then examined microscopically with a magnification of 300 to 400. As is always the case in examining unstained objects, the concave mirror is used, and the *Abbé* condenser thrown out.

Microchemical reactions must frequently be used for the identification of amorphous and crystalline salts. These are carried out by placing a drop of the reagent at one side of the cover-glass, and drawing it through under the glass by means of a piece of filter-paper, which is placed at the opposite side.

Microscopical Examination

Urinary sediment is composed of unorganized and organized solids.

The unorganized constituents are the salts which are precipitated from the urine, and which appear in the sediment either in amorphous or crystalline form.

No attempt should be made to divide the salts according to the reaction of the urine in the sediment of which they are usually found, since most of them may be contained in the sediment of acid, amphoteric, and alkaline urine. Uric acid, for example, appears principally in acid, ammonium magnesium phosphate in alkaline urine; nevertheless, they may appear together in alkaline urine, when, in the early stage of alkaline fermentation, the uric acid crystals are not yet completely dissolved, while the triple phosphate crystals have already been precipitated. In the description of the various salts the reaction of the urine in which they are usually found will be mentioned.

Uric Acid (Plate V, Fig. I).—Crystals of uric acid appear principally in the sediment of acid urine, more rarely in that of amphoteric, and only under special conditions in that of alkaline urine. They sometimes appear singly, and sometimes in great quantity, and then frequently cling to the sides and bottom of the vessel, and can usually be recognized macroscopically by their crystalline appearance and their yellow or red-brown color.

Microscopically, uric acid crystals appear almost always brown or yellow; colorless crystals are very rarely seen. They vary greatly in form and size. They appear in the form of whetstones and of spindles, which, lying crosswise over each other, resemble glands and rosettes, as hexagonal plates, and in cask or barrel forms. Spear and needle forms are also seen, arranged in sheaves or tufts. Dumb-bell and hour-glass forms are more rarely seen. These various forms of crystals, which frequently appear side by side, can always be traced back to a common form—the rhomboid plate. If two opposite angles of the plate are rounded, the whetstone form is produced;

if they are cut off by straight lines, the hexagonal forms are produced; if the corners are drawn out to an angle, the needle or spear forms are produced; if the crystals are piled upon one another, the cask and barrel forms are produced.

Uric acid crystals can usually be recognized at once by their color, which they owe to the urinary pigments extracted at the time of their crystallization. The colorless four to six sided plates, in which uric acid may crystallize, resemble cystin crystals, but can be distinguished from them by their chemical behavior.

Microchemical Reactions:

1. Uric acid gives the murexide test (cf. page 207).

2. If a little sodium hydrate is allowed to run under the cover-glass, the crystals of uric acid are dissolved, to be reprecipitated upon the addition of hydrochloric acid.

Amorphous Urates (Fig. 25).—These consist of the urates of sodium, potassium, calcium, and magnesium, and form a sediment of acid urine. Macroscopically, they appear as a clay-colored, yellow, or brick-red sediment, which is often precipitated in large quantity from concentrated acid urine, and from urine exposed to the cold (*sedimentum lateritium*). The color of this sediment is due to the normal pigments of the urine, which the urates, like uric acid, extract when precipitated.

Microscopically, they appear as small, amorphous, brownish-yellow, more rarely colorless granules, which usually lie together in mosslike groups of varying size, often in such thick masses that they cover the entire field, hiding all other solid elements. To render these latter visible it is necessary to dissolve the urates. This is most easily accomplished by filling the centrifuge-tube, containing the sediment, with lukewarm physiological salt solution, dissolving the urates by shaking, and centrifugalizing at once, before they can be reprecipitated by the cooling of the mixture. Occasionally urates form peculiar cylindrical figures, urate casts, which must not be confused with granular casts; frequently they are seen lying upon epithelial cells and true casts.

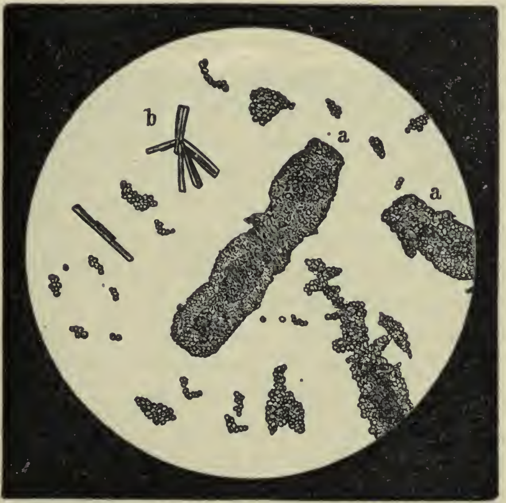

FIG. 25.—*a*, Urate casts; *b*, neutral calcium phosphate.

Microchemical Reactions:

1. Urates are dissolved by heating, and reprecipitated by cooling.

2. They are dissolved by the addition of hydrochloric and acetic acids, uric acid crystals being after a time precipitated from the solution in the form of rhomboid plates.

3. The murexide test is positive.

Ammonium Urate (Plate VI, Fig. J).—Ammonium urate is the only salt of uric acid which is found in the sediment of alkaline urine. It is found rather frequently in neutral and acid urine in children, especially newborn and nursing children, much more rarely in adults.

The presence of ammonium urate cannot be detected macroscopically. Its microscopical appearance is, however, very characteristic; usually it appears in the form of brownish-yellow spheres, which may lie singly, in pairs, or in large groups. These spheres frequently show spicules, which, according to their size and number, give a varied appearance to the crystals. Thus, crystals of thorn-, apple-, mace-, mite-, and turnip-form are produced. Crystals of ammonium urate are rarely colorless. They then appear as dumb-bells or as tufts of needles. The simultaneous appearance of typical brown spheres, as well as microchemical reactions, make it possible to easily recognize these rarer crystals.

Microchemical Reactions:

1. Crystals of ammonium urate are dissolved by heating, and are reprecipitated by cooling.
2. Upon the addition of acetic acid they are dissolved, and in their place crystals of uric acid are formed.
3. They are dissolved by potassium hydrate with the formation of gas (ammonia).
4. Like all urates, they give the murexide reaction.

Calcium Oxalate (Fig. 26).—Crystals of calcium oxalate appear in the sediment of acid, amphoteric, and faintly alkaline urine. When precipitated in large quantity, they form a grayish-white, flaky sediment. They appear usually as colorless, highly refractive octahedra, the so-called envelope-forms, of varying size. Very small crystals, whose envelope-form can often be detected only by careful focusing, are seen, especially when calcium oxalate is precipitated in large quantity. Even the most minute, punctiform crystals attract attention, however, by their characteristic glistening appearance, often resembling minute fat drops, from which they are distinguished by microchemical reactions. (Fat is dissolved by the addition of ether.)

Calcium oxalate crystallizes in hour-glass, dumb-bell, or biscuit form, more rarely than in octahedra. The high refractive power of these objects, whose surface is slightly striated, the simultaneous presence of envelope forms, and, finally, their behavior toward chemical reagents, make it possible to recognize them as crystals of calcium oxalate. In icteric urine they are, like other solid constituents (epithelium cells, casts, etc.), frequently yellow.

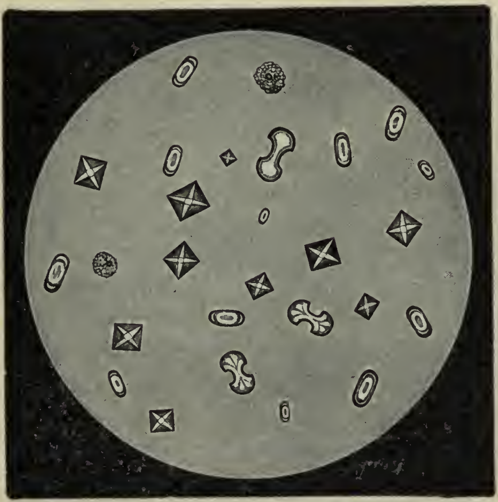

FIG. 26.—Calcium Oxalate.

Crystals of calcium oxalate are characterized chemically by their insolubility on heating, and in acetic acid, and their easy solubility in hydrochloric acid. If ammonia or potassium hydrate is added to the solution in hydrochloric acid, the calcium oxalate recrystallizes in octahedra.

Neutral Calcium Phosphate (Dicalcium Phosphate—Fig. 27).—This appears in the sediment of slightly acid, amphoteric, and faintly alkaline urine. Neutral calcium phosphate crystallizes, usually in long, glistening, prismatic, cuneiform crystals, which may be seen singly, but are usually arranged in bundles of varying thickness, or in rosettes. In the latter case the arrow-pointed heads are usually directed toward the centre. Dicalcium phosphate also crystallizes in plates, and, in rare cases, in tufts of needles, which resemble tyrosin crystals in appearance, but can be distinguished from them by their microchemi-

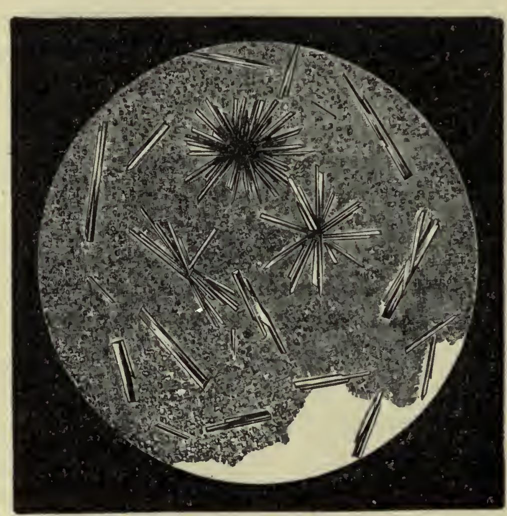

FIG. 27.—*a*, Crystals of neutral calcium phosphate; *b*, amorphous phosphates and carbonates.

cal reactions. Crystals of neutral calcium phosphate are completely dissolved by treating with acetic acid.

Calcium Sulphate.—Crystals of calcium sulphate are very rarely detected in the sediment of the urine. They appear only in highly acid urine, in which, if present, they frequently form a thick white precipitate.

Microscopically, they appear as long, colorless needles, or as slim prisms, with oblique bases, usually arranged in rosettes. The following microchemical reaction prevents confusion with the crystals of neutral calcium phosphate, which resemble them closely. Crystals of calcium sulphate are insoluble in acetic acid, and soluble with difficulty in hydrochloric acid.

Calcium Carbonate.—Calcium carbonate appears most frequently in the sediment of alkaline, much less frequently in that of amphoteric or faintly acid urine. It usually appears together with amorphous phosphates, from which it cannot be distinguished macroscopically. Microscopically, it appears in the form of small grayish-white granules or spherules, which frequently lie upon one another. Their microchemical behavior is characteristic. Upon the addition of dilute mineral acids, the carbonates are dissolved with the liberation of CO_2, so that the entire microscopical field is covered with minute air-bubbles.

Amorphous Earthy Phosphates (Calcium and Magnesium Phosphates—cf. Fig. 27).—These are most frequently precipitated from alkaline, but may appear in the sediment of amphoteric or faintly acid urine. They form a fine, flaky, grayish-white, easily mobile precipitate.

Microscopically, they appear as finely granular, colorless masses, which can be easily distinguished by microchemical reactions from other amorphous sediments resembling them in appearance. The earthy phosphates are dissolved upon the addition of acetic acid without the liberation of gas, but are not dissolved by heating.

Ammonium Magnesium Phosphate (Triple Phosphate—Fig. 28).—This appears principally in the sediment of alkaline urine, frequently together with amorphous phosphates and carbonates, as well as in the purulent sediment of alkaline urine. It is, however, occasionally found in amphoteric and faintly acid urine in commencing alkaline fermentation.

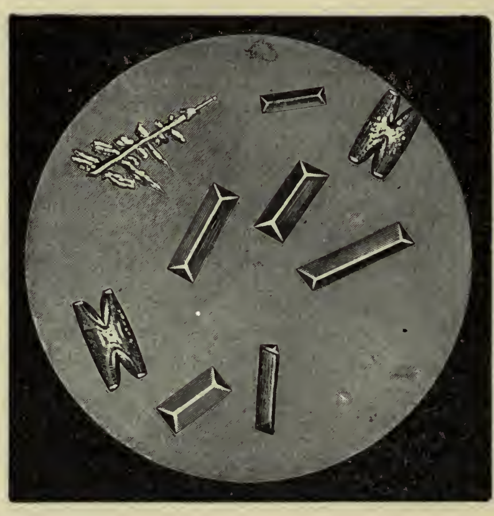

Fig. 28.—Triple Phosphate (Ammonium Magnesium Phosphate).

Triple phosphate forms rhomboid, clear prisms of very characteristic appearance. Usually they appear in the coffin-lid form, more rarely as penniform or fernlike structures. Now and then very grotesque crystals are produced by combinations of these forms, which, however, can be identified as triple phosphate microchemically, by their easy solubility upon the addition of acetic acid. This characteristic reaction prevents confusion of triple phosphate crystals with the large envelope forms of calcium oxalate, which occasionally resemble them closely.

Magnesium Phosphate Crystals.—These are found in very rare cases in the sediment of alkaline urine in the form of glistening, elongated, rhomboid plates, which are easily soluble in acetic acid. They are also frequently seen in the film which covers the surface of alkaline urine.

Leucin and ***Tyrosin*** (Fig. 29), which are usually found together, do not, in contradistinction to the above-described forms of crystals, appear in normal urine. Their appearance has been observed in acute yellow atrophy of the liver, phosphorus-poisoning, and, more rarely, in infectious diseases, as typhoid and variola, and in serious diseases of the blood.

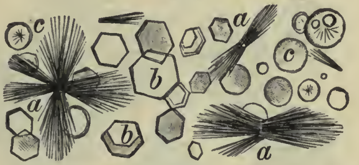

FIG. 29.—*a*, Tyrosin; *b*, cystin; *c*, leucin.

The detection of leucin crystals succeeds, as a rule, only after the evaporation of the urine, and their precipitation with alcohol. In cases, however, in which leucin is present in great quantity, it crystallizes spontaneously if a drop of the urine is allowed to slowly evaporate on a slide. Tyrosin is soluble with more difficulty than leucin, and is also usually present in the urine in greater quantity. It is often precipitated, therefore, spontaneously after the urine has stood awhile. Tyrosin crystals, which, like those of leucin, are usually greenish-yellow, form tufts composed of very fine needles, and leucin crystals form spheres, which usually allow both a radial and concentric striation to be seen. Small spheres are frequently seen attached to the large ones.

Microchemical Reactions.—Leucin is soluble in acids and alkalies, insoluble in alcohol and ether. Crystals of ammonium urate, with which leucin crystals can be confused, are distinguished from them by the appearance of uric acid crystals after they have been dissolved in acetic acid.

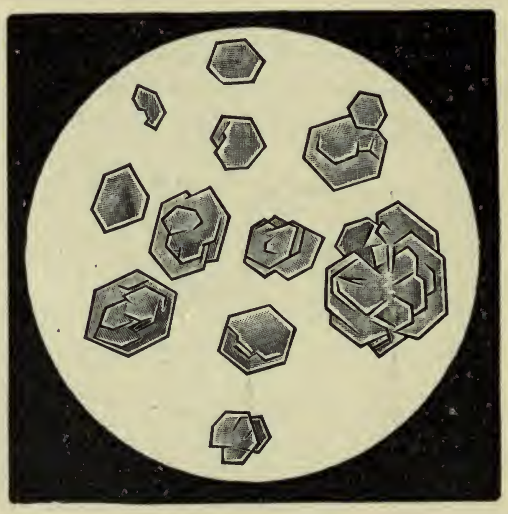

FIG. 30.—Cystin Crystals.

Tyrosin is insoluble in acetic acid, alcohol, and ether; soluble in dilute mineral acids, alkalies, and ammonia.

Cystin (Fig. 30) also does not appear in the urine under normal conditions. It appears in the sediment in the rare cases of cystinuria, in which, from causes not yet thoroughly explained, cystin is excreted in the urine. Cystin crystallizes in characteristic, colorless, hexagonal plates, which are frequently arranged in strata. Cystin is, in contradistinction to uric acid, soluble in hydrochloric acid and ammonia; it is insoluble in acetic acid. If acetic acid is added to the ammoniacal solution, or if the ammonia is allowed to slowly evaporate, the cystin crystals are reprecipitated in the form of hexagonal plates.

Hippuric Acid (Fig. 31).—Crystals of hippuric acid appear very rarely in the sediment of the urine. Hippuric acid crystallizes in colorless needles and rhomboid prisms, which may have a stellate arrangement. It is insoluble in acetic acid.

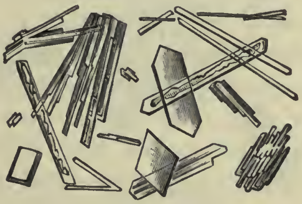

FIG. 31.—Crystals of Hippuric Acid.

Cholesterin appears also very rarely in the sediment of the urine. Cholesterin crystals appear as colorless plates, which frequently lie in strata, and have notched corners. For microchemical reactions, cf. p. 93.

Xanthin, though normally present in the urine, has as yet been found in the sediment in very few cases. It forms whetstone-shaped crystals, which are, in contradistinction to uric acid, readily soluble in dilute ammonia and on heating.

Of the pigments appearing in the urine, bile-pigment and blood-pigment, as well as indigo, occasionally form amorphous and crystalline precipitates.

Bilirubin.—Bile-pigment may be precipitated as orange-colored, amorphous granules, or as yellow crystals, in the form of needles and rhomboid plates, in icterus neonatorum and icterus gravis of adults, especially when the urine is highly acid. The granules and crystals are often found in epithelial cells, leucocytes, or fat drops.

Haemoglobin (Plate VII, Fig. K).—In hemorrhage from the kidneys and the urinary passages and in hæmoglobinuria, blood-pigment is frequently precipitated in the form of reddish to brownish-yellow granules and flakes. Blood pigment may be precipitated in great quantity, especially in severe cases of hæmoglobinuria, and then often forms cylindrical objects (pigment casts). Blood-pigment appears more rarely in the form of hæmatoidin crystals. These resemble the above-described bilirubin crystals, and are frequently considered as identical with them.

Indigo.—Indigo-blue is occasionally formed in alkaline decomposition of urine, rich in indican, by the oxidation of the indican. The blue crystals, often noticeable macroscopically because of their color, appear as small rhomboid plates or tufts of needles, which are dissolved in chloroform, coloring it blue.

Fat and Fatty-Acid Needles (Fig. 32).—When fat is found in the urine it must always be remembered that its presence may be due to accidental contamination, by means of greased catheters, suppositories, greasy receptacles, etc. Under pathological conditions fat appears in the urine in macroscopical quantities only in the rare cases of lipuria and chyluria.

Microscopically, fat appears in the form of highly refractive drops and granules with sharply defined, dark margins, either floating free in the liquid, or lying upon other solid elements, as, for example, casts, or as the product of fatty degeneration of the protoplasm lying within the cells. Often the latter are so filled with fat globules that the nucleus is invisible, and the cell resembles a colostrum corpuscle (fat-granule cells—Fig. 32).

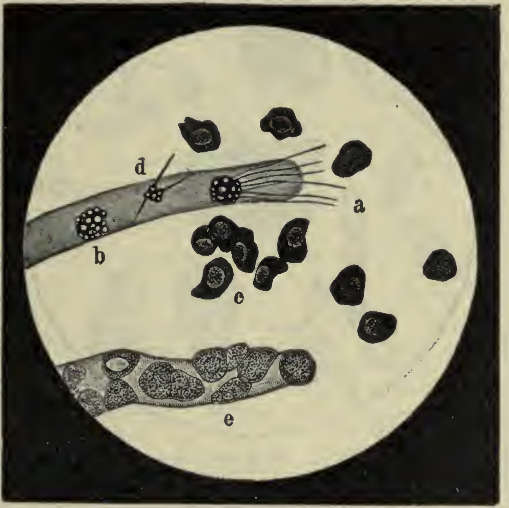

FIG. 32.—*a*, Fatty-acid needles; *b*, fatty degenerated renal epithelial cells (fat-granule cells); *c*, renal epithelial cells; *d*, hyaline cast; *e*, cast covered with renal epithelial cells.

Fatty-acid crystals are occasionally seen together with fat drops. They appear as straight or wavy needles, which frequently have a stellate arrangement, or radiate from a fat drop.

Fat is stained black by a 1 per cent. solution of osmic acid, and bright red by a saturated alcoholic solution of

Sudan III. It is characterized chemically by its solubility in ether, chloroform, and carbon bisulphide.

Organized Sediments

Epithelium.—The epithelial cells, which are found in urinary sediment, have a varied appearance (Fig. 33).

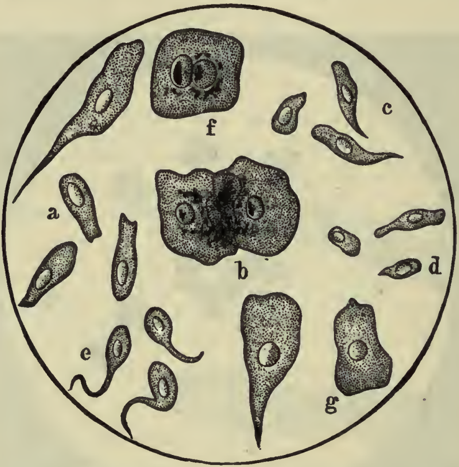

FIG. 33.—Epithelial Cells. *a,* From the male urethra; *b,* from the vagina; *c,* from the prostate; *d,* from *Cowper's* glands; *e,* from *Littre's* glands; *f,* from the female urethra; *g,* from the bladder (according to *Loebisch*).

They may be divided, according to their origin, into three groups:

1. Epithelium from the urinary passages.
2. Renal epithelium.
3. Epithelium from the genitalia (prepuce, vagina, vulva).

Careful histological investigations have shown that the entire urinary tract, from the pelvis of the kidney to the fossa navicularis urethræ, is lined with stratified epithelium, which, with the exception of slight local differences, is of the same type. The superficial layer usually consists of polygonal, mono- or polynuclear, flat epithelial cells, which have indentations on their under surface. The cells of the second layer, which is usually composed of several rows of oval, pear-shaped, tailed cells, fit into these indentations. The lowest layer consists of small, polygonal, or round cells with large nuclei. The anterior portion of the urethra to the fossa navicularis is lined with stratified squamous epithelium, while the superficial layer of the pars cavernosa and membranacea urethræ consists, according to most authors, of cylindrical cells.

Any of these forms of cells may be found in the sediment of the urine in varying quantity, without it being possible to tell from their appearance from what portion of the urinary tract they come. Their examination can only reveal which layer of the epithelial lining is in the process of desquamation. The wide-spread idea that the appearance of tailed epithelial cells in the sediment of the urine depends upon the existence of pyelitis must, therefore, be discarded as erroneous, since histological investigation has shown that this form of cell is in no wise peculiar to the pelvis of the kidney.

Normal urine always contains in the nubecula, or in its precipitate, occasional flat epithelial cells (Fig. 33). In inflammatory processes of the urinary tract numerous epithelial cells of varying form appear in the sediment in addition to the other products of inflammation. Epithelial cells may show all kinds of degeneration: they may be swollen, the nuclei indistinct, the protoplasm filled with vacuoles, or in the process of fatty or hyaline degeneration.

Renal Epithelial Cells (Fig. 32).—These appear as round or cuboid, sharply bordered cells with large, often vesicular, nuclei. The protoplasm is finely granular, and usually in a state of more or less marked fatty degeneration. They are slightly larger than leuçocytes, from which they can often be distinguished only by their distinct nucleus and sharply defined contour. If they lie singly they are not always easy to recognize, because of their close resemblance to the epithelial cells of the lowest layer of the urinary passages. Only their characteristic arrangement in epithelial threads, or the simultaneous presence of casts, upon which they frequently lie, identifies them as renal cells. In icteric urine they are frequently stained yellow.

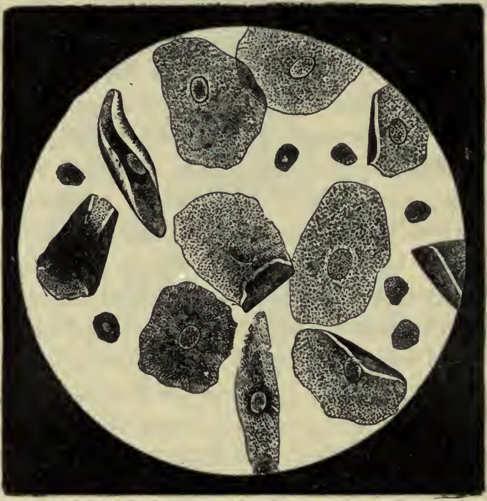

FIG. 34.—Squamous Epithelial Cells.

The presence of renal epithelium in the sediment always indicates disease of the kidney.

Epithelial Cells from the Genitalia (Fig. 34) appear in the urine as large squamous cells, and come in the male from the prepuce; in the female from the vulva and vagina. These cells frequently appear folded and with curled edges. In the urine of women, in which they are present normally in great numbers, white flakes are often seen with the naked eye, which are found microscopically to be continuous membranes of large squamous cells.

Leucocytes (Pus-corpuscles—Plate IX, Fig. M).—The sediment of normal urine contains a few isolated leucocytes, which, however, have no diagnostic significance. In the urine of women with leucorrhœa, leucocytes appear in great numbers without indicating disease of the urinary tract. Leucocytes appear in the urine in great numbers as constituents of pus. The urine then appears more or less turbid, and on standing a sediment is formed, the character of which differs with the reaction of the urine. In acid, amphoteric, and faintly alkaline urine, pus forms a non-transparent, flaky, gray, or yellowish-white sediment, which appears homogeneous, or contains threads or clumps of blood, crystals, etc. In contradistinction to phosphatic sediment, which resembles it in appearance, purulent sediment is insoluble in acetic acid, and upon the addition of caustic potash (*Donné's* test) becomes a glairy, mucoid, stringy mass, which represents the purulent sediment of strongly alkaline and ammoniacal urine. When such a sediment is poured from the vessel it frequently slides out as a gelatinous coagulum.

The *microscopical* picture which leucocytes present also depends upon the reaction of the urine. In acid and faintly acid urine they appear as round, colorless cells with granular, refractive protoplasm. They have one or more nuclei, which are only clearly seen after the addition of reagents. If a drop of acetic acid is allowed to run under the cover-glass, the granulation disappears, the protoplasm becomes transparent, and one or more irregular, often horseshoe-shaped, nuclei with nucleoli are visible.

In strongly alkaline and ammoniacal urine pus-corpuscles are usually in a state of degeneration. They are glassy, swollen, transparent, and the granules have disappeared, or as a narrow border surround a clear central zone in which the nucleus is still visible. As the degeneration advances, the contour of the individual cells fades, the nuclei become indistinct, and the leucocytes finally form a granular detritus, in which isolated free nuclei and but few unaltered cells are visible.

Their insolubility in acetic acid prevents confusion of these products of the decomposition of leucocytes with amorphous phosphates.

Red blood-corpuscles appear as round, biconcave, yellow discs, lying singly or in groups. In renal hemorrhage they appear arranged in cylinders (blood-casts), or lying upon casts. Frequently they cover the entire field, and completely obscure the other solid constituents. The latter can be seen only after the red blood-corpuscles have been dissolved by allowing a drop of distilled water or dilute acetic acid to run under the cover-glass.

Erythrocytes frequently show alterations in form and color, depending upon the concentration of the urine, its reaction, and the length of time that they have been present in it. In faintly acid urine they remain unaltered for some time, while in concentrated and highly acid urine they appear shrivelled and crenated. In the presence of a strongly alkaline reaction they degenerate, and finally become decomposed, forming clumps and flakes consisting of blood-pigment (cf. hæmoglobin).

After long contact with the urine, and in very dilute urine, their pigment is extracted, they swell up, and appear as colorless, annular bodies (shadow corpuscles), which are recognized with difficulty, especially when they are isolated.

Red blood-corpuscles may occasionally be confused with *yeast cells.* For differentiation, a drop of 1 to 2 per cent. acetic acid is added; red blood-corpuscles are almost completely dissolved and become invisible, while yeast cells remain unaltered.

Frequently in bloody urine clots are found, which can be detected with the naked eye. They differ widely in their macroscopical appearance; they appear sometimes as irregular clumps or flakes, sometimes as thready, rod-shaped, or vermiform objects, which may be as thick as a finger and several centimetres in length. They may be red, reddish-brown, or blackish-brown, or frequently grayish-white. The latter is true of coagula which have been in the urine a long time.

The long, slim clots have diagnostic significance. Since they are thought to be formed in the ureter, their appearance suggests that the seat of the hemorrhage is in the ureter itself, in the kidney, or its pelvis. The possibility that such coagula may owe their form to their passage through the urethra must, however, be considered. The form of clot, therefore, does not suffice to determine the location of the hemorrhage; on the contrary, all the accompanying symptoms of the hæmaturia must be considered.

Microscopically, blood-coagula appear as a net-work of fibrin, whose meshes are filled with a varying number of unaltered and altered blood-corpuscles. Blood is detected microchemically by the tests described on p. 66.

Fibrin (Plate IX, Fig. N).—In addition to the above-described blood-clots, whose framework is composed of fibrin, structures composed entirely of fibrin appear in the urine following hæmaturia.

Macroscopical quantities of fibrin are passed in the urine in the rare cases of so-called fibrinuria and chyluria, in which they form white, gelatinous clots either before or after the urine is passed.

Microscopically, fibrin-clots are found to be composed of bundles of parallel, highly refractive, white, or reddish-yellow fibres. In doubtful cases they can be recognized by means of *Weigert's* fibrin stain (Fig. 53).

Casts (Plate VIII, Fig. L, and Plate X, Fig. O).—Casts are microscopical, cylindrical structures of varying length and thickness, with sharply defined, parallel sides, and rounded ends. They are sometimes straight and sometimes wavy, and are often bent or indented. Frequently one end of the cast appears to have been broken off. Fragments are also often seen, which can be recognized only by comparison with intact casts. Casts are renal in origin, and owe their form to the urinary tubules, from which they are washed by the urine.

The following varieties are distinguished: (1) Casts composed of cells. (2) Granular casts. (3) Hyaline casts. (4) Waxy casts.

Casts of the first group are designated, according to the form of cell, as epithelial, blood, and leucocyte casts.

The renal epithelial cells, of which the epithelial casts are composed, are almost never unaltered, being usually in a state of granular or fatty degeneration.

If the degeneration is more advanced, the outline of the cells is obliterated, their nuclei are difficult to recognize, or have entirely disappeared, and finally their epithelial character is completely lost, and the picture of the granular cast is produced. Frequently one-half of a cast has still a distinct epithelial appearance, while the other appears granular.

Granular casts have a granular surface, which gives them a dark appearance. These granules, which, depending upon their origin, may consist of albumin or fat, are sometimes small and sometimes large, so that a distinction is made between finely and coarsely granular casts. If the granules consist principally of minute fat droplets, the casts are called fat-granule casts, and attract notice by their glistening appearance, which they owe to the high refractive power of the fat.

In the urine of women numerous long, granular epithelial cells from the external genitalia are often seen, and make the recognition of granular casts more or less difficult, depending upon the experience of the observer; the usually distinct nucleus of the epithelial cells, however, prevents confusion.

Hyaline casts have a pale, homogeneous, transparent, basic substance, whose margin, however, is always distinct. These structureless and colorless objects are frequently so delicate that they can be recognized only with difficulty. Their detection is simplified by the deposits which they frequently have upon them. Cellular elements, as renal epithelium, red and white blood-corpuscles, as well as fat drops, granular detritus, micro-organisms, and salts, frequently entirely, or partially, cover them.

To simplify the detection of hyaline casts they may be stained, by adding to the sediment a few drops of *Lugol's* solution, or a saturated watery solution of picric acid. Thin, watery fuchsin or methylene-blue solutions may also be used for this purpose.

Waxy casts have, like hyaline, a homogeneous basic substance, but are broader, larger, and of tougher consistency.

They are waxy, moderately refractive in appearance, yellow in color, and frequently show deep indentations; occasionally very broad, short forms are seen.

Cylindroids (Fig. 35), which are found both in normal and pathological urine, must be distinguished from true casts. They are most easily confused with hyaline casts. In contradistinction to the latter, their basic substance is not homogeneous, but usually shows a distinct longitudinal striation. In addition they have, as a rule, frayed or forked ends.

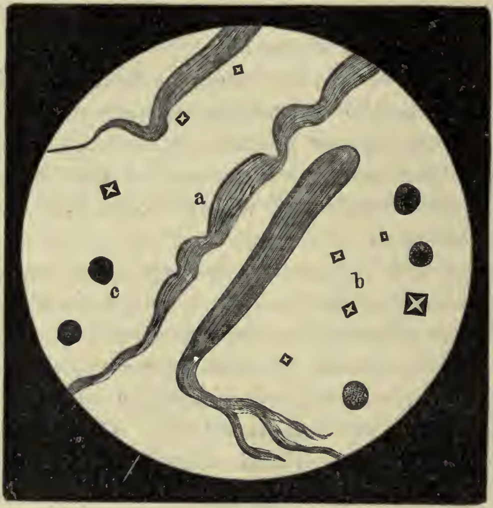

FIG. 35.—*a*, Cylindroids; *b*, crystals of calcium oxalate; *c*, leucocytes.

Clumps of bacteria, which resemble granular casts in form, are occasionally found in the sediment, and are called bacterial casts. Examination with the high power and staining with a dilute watery solution of fuchsin or methylene blue will identify these structures.

Fragments of Tissue.—Fragments of tissue are rarely found in the urine. They may be easily overlooked in turbid urine, particularly when it contains blood or pus. To prevent this, such urine should be poured into a flat dish, in which it can be conveniently examined. The fragments are removed and examined separately. Fragments of tissue are passed in the urine in tumors of the kidney and urinary passages, in severe septic cystitis, which has caused gangrene of the mucosa of the bladder, as well as in pyelitis. When tumors of the neighboring organs have extended into the urinary tract, particles of them may, of course, be passed in the urine. Fragments of tissue must be especially examined histologically.

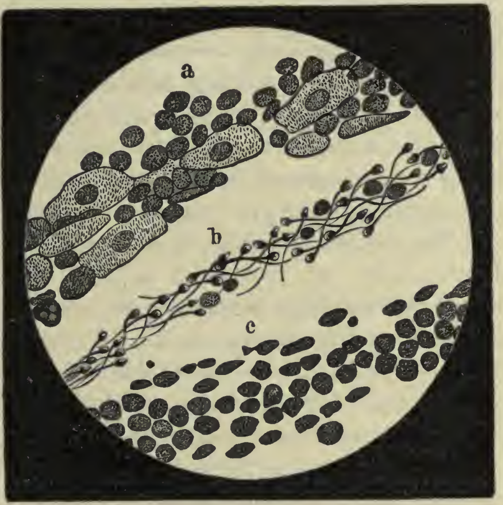

FIG. 36.—*a*, Urinary filament, composed of pus-corpuscles and epithelial cells; *b*, urinary filament, composed of spermatozoa and occasional leucocytes; *c*, urinary filament composed of pus-corpuscles.

Urinary Filaments (Urethral Threads) (Fig. 36).—By urinary filaments are meant small threads or flakes which are passed in the urine as products of the purulent or mucoid secretion of the urethra and genital glands. They are of varying size, often 1 to 2 centimetres in length, and appear muco-gelatinous, or yellow and non-transparent, but all stages between these two types are seen.

Filaments are present in the urine in chronic gonorrhœa (gonorrhœal threads), also in the urine of neurasthenic patients having urethrorrhœa, and occasionally in the first morning urine of healthy persons.

The microscopical picture presented by the urethral filaments is the same in the last two cases. They are composed of a homogeneous, transparent basic substance, in which a varying quantity of epithelial cells, a few leucocytes, and frequently amorphous and crystalline salts are embedded.

The urethral threads in gonorrhœa are composed either of thick clumps of pus-corpuscles or of both epithelial cells and pus-corpuscles, sometimes more of one and sometimes more of the other, or of epithelial cells alone. In cases in which ejaculation of semen has preceded the passage of the urine, as well as in the urine of persons suffering from spermatorrhœa, spermatozoa are also present in the filaments.

Microscopical examination shows that the macroscopical appearance of urethral filaments depends upon their richness in cells. The fewer the cells contained the nearer they approach the type of the muco-gelatinous filaments.

For microscopical examination of the filaments the first morning urine is best used, from which only the first 10 to 15 cc are collected, since the filaments, particularly the yellow, are usually very fragile, and are easily dissolved by a large quantity of urine. They are removed with a pipette or a bent needle, and carefully spread on a slide.

Secretion from the Genital Glands (Plate X, Fig. P).—Spermatozoa are frequently present in the sediment of the urine. They are present in the urine following coitus and pollutions, in diseases of the genital organs, as well as following convulsions, and in severe febrile diseases, particularly typhoid fever. They appear sometimes singly, sometimes in great quantity, and frequently arranged in filaments. Spermatozoa may also be found in the urine of women passed after coitus.

Occasionally they are still lively, but very frequently motionless. Large round cells with distinct nuclei are also sometimes seen enclosing spermatozoa. Delicate, pale cylindrical objects, with a homogeneous basic substance, are often seen. These, the so-called testicular casts, come from the tubules of the testicle, and resemble hyaline casts. They are distinguished from true hyaline casts by the simultaneous presence of spermatozoa, which frequently lie upon them.

Prostatic Secretion is mixed with the urine in diseases of the prostate and following its massage. Numerous small, glistening granules, called lecithin granules, are then also present in the sediment, and in addition round or angular objects with a distinct concentric striation, which are called prostatic bodies, or, since they resemble starch granules, *Corpora amylacea.*

Animal Parasites.—Of the animal parasites which may appear in the urine the echinococcus is of special interest, since the others either are not observed in our latitude or are merely present accidentally, and have no pathognomonic significance.

Portions of the *echinococcus* (Plate XI, Fig. Q) appear in the urine when the echinococci are located in the urinary tract, or when an echinococcus cyst has ruptured into it from the neighboring tissues. Entire cysts which may be passed in great numbers are then found, as well as the characteristic hooklets and shreds of membrane, which can be easily recognized by their distinct stratification.

The following parasites are more rarely found: Embryos of the *Filaria sanguinis* (in tropical chyluria), eggs of the *Eustrongylus gigas*, eggs of the *Distoma hæmatobium* (in bilharziosis).

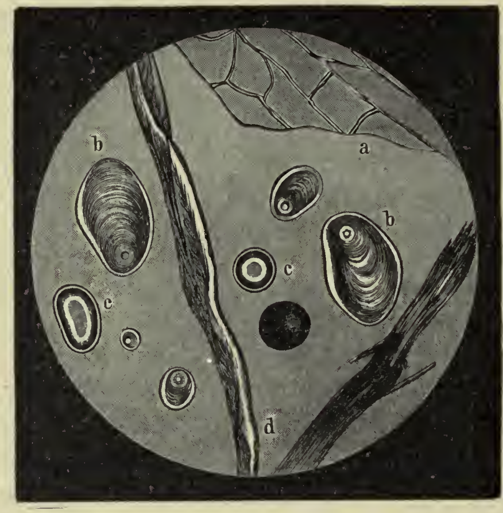

Fig. 37.—*a*. Vegetable cells; *b*, starch granules; *c*, air-bubbles; *d*, vegetable fibres.

The infusoria, *Cercomonas urinarius*, and *Trichomonas vaginalis*, which occasionally appear in the sediment of the urine have no significance. Occasionally amœbæ, the larvæ of flies, and pediculi pubis, are found in the sediment of the urine as accidental constituents.

Substances Found in the Sediment Due to Contamination of the Urine (Fig. 37).—The presence in the sediment of food particles, vegetable cells, muscle fibres, etc., indicates the contamination of the urine with fæces. If this contamination is due to a recto-vesical fistula, the urine shows simultaneously evidences of severe cystitis. Constituents of the fæces found in the sediment are usually, however, due to contaminated urinary receptacles.

Sputum, hair, vegetable and animal fibres, starch granules, fat, and fungi may also be adventitious constituents of the urinary sediment.

VIII. Bacteriological Examination of the Urine

Collection of the Urine for Examination

The urine is best collected for bacteriological examination by means of a sterile catheter, following a thorough cleansing of the external genitalia and irrigation of the anterior urethra, which normally possesses a luxuriant bacterial flora. The urine first passed, which, in spite of the irrigation, may contain micro-organisms or secretions, which have been carried by the catheter from the urethra into the bladder, is allowed to escape, and the following portion collected in a sterile receptacle. If for any reason the urine cannot be obtained per catheter, the external genitalia are cleansed, the urethra irrigated, and the first portion of the urine allowed to escape, which cleanses the urethra still further, and the second portion used for examination. The urine should be examined as soon as possible after its evacuation, since the micro-organisms present usually multiply very rapidly.

Preparation of the Urine for Examination

In most cases it is advisable to centrifugalize the urine in sterile tubes, and to use the sediment so obtained for examination. When, however, the urine is very rich in bacteria, which can be determined by examining a hanging drop, it is sufficient to take a drop of it for examination, as, for example, in the so-called bacteriuria. Occasionally, in the latter case, no sediment is obtained by centrifugalization. To obtain a sediment from such urine, absolute alcohol may be added, which lowers the specific gravity of the liquid, with the result that, when centrifugalized, the solid constituents are precipitated. To obtain a sediment in ammoniacal urine, it is often necessary to heat it in a water-bath with dilute potassium hydrate. In the two latter cases the precipitate is, of course, unfit for cultural use. In urine rich in urates the salts are first dissolved by slight heating; for this purpose the urine may be placed for a few minutes in an incubator at 37° C.

Method of Examination

Urine is examined bacteriologically by means of stained smears, cultural procedures, and animal inoculation.

Smears are made in the usual manner. In the presence of a large amount of crystalline salts they are, however, best fixed in absolute alcohol (ten minutes); in the presence of fat or blood in alcohol and ether (three minutes). The smears are stained with dilute borax methylene blue (1 : 9) two minutes, without heating, according to *Gram*, and according to one of the methods for detecting tubercle bacilli.

Cultural Procedures.—Agar is best used for isolating the bacteria which appear in the urine. Special culture media are, however, necessary for the detection of tubercle bacilli and gonococci.

Animal inoculation is used, as a rule, only in the diagnosis of tuberculosis. Guinea-pigs are used as test-animals, and are inoculated with the sediment obtained by centrifugalizing the urine in the manner described under examination of the sputum.

The pathogenic bacteria of importance in examination of the urine are the bacilli belonging to the group of *B. coli*, tubercle bacilli, staphylo-, strepto-, and gonococci, typhoid bacilli, *Proteus vulgaris*, and *B. pyocyaneus.*

Frequently a mixture of different organisms is seen in the stained smears. It is then impossible to tell which bacteria should be considered as the exciting cause of the disease. Frequently the picture which the bacterial flora presents in these cases is not constant, but varies with the different examinations. Such a condition is caused by the bacteria of decomposition, which have become secondarily located in the diseased bladder, and therefore the isolation and identification of the various bacteria have merely a scientific, and no diagnostic, value.

Bacterium Coli (Plate XI, Fig. R) is by far the most frequent exciting cause of cystitis and pyelitis; bacteriuria caused by it is often observed. The urine is acid in reaction so long as none of the bacteria of decomposition have gained entrance to the bladder. Under the name of *B. coli* is included a group of bacilli whose type is the *B. coli*, cultivated by *Escherich* from the intestine of the nursing child. The different members of this group vary in their morphological and biological characteristics, depending, to a certain extent, upon external conditions; but they also possess a number of constant characteristics. Among the latter are their luxuriant growth upon all the usual culture media, their very slight tendency to liquefy gelatine and to form spores, and the fact that they are decolorized by *Gram*. The different members vary in their ability to ferment sugar, coagulate milk, form indol, etc.

B. coli appears in the stained smear made from urinary sediment as a plump, straight rod with rounded ends, and of varying length. The bacteria lie singly in pairs or in groups and frequently form chains; more rarely they lie within the cells. They are decolorized in specimens stained according to *Gram*.

They are easily cultivated on agar. After twenty-four hours' growth at 37° C. grayish-white colonies have developed. Concerning the identification of the cultivated bacteria by means of transplantation on litmus-whey, neutral-red agar, milk, etc., cf. the Examination of the Fæces for Typhoid Bacilli (p. 108).

Staphylococci and ***Streptococci*** appear as independent exciters of disease more rarely than *B. coli*, but they appear frequently as producers of mixed infection in cystitis and pyelitis.

Both varieties of cocci stain according to *Gram*, and are, therefore, especially conspicuous in smears so stained. Staphylococci frequently lie within the cells. For differential diagnosis only gonococci come into consideration, from which staphylococci and streptococci are easily distinguished by their form, staining characteristics, and the ease with which they can be cultivated upon the usual culture media. In regard to their cultural characteristics, cf. Examination of Sputum.

Tubercle Bacilli (Plate XII, Fig. S).—Urine passed in tuberculosis of the urinary tract is acid in reaction so long as the bacteria of decomposition have not gained entrance to the bladder. Acid purulent urine, in which no bacteria are detected either in stained smears or by means of cultural procedures, always arouses suspicion of tuberculosis.

Specimens are stained for tubercle bacilli in the same manner as in the examination of the sputum. Tubercle bacilli in the urine do not differ in appearance from the picture which they present in the sputum.

The quantity in which they appear in the urine varies greatly. In tubercular cystitis they are often present in great numbers, lying either singly or in groups, and frequently in characteristically plaited or **S**-shaped arrangement. In other cases, especially in tuberculosis of the kidney, their detection is extremely difficult, and a great number of specimens must be examined before the first bacillus is found. When the attempt to detect the bacilli in the sediment of urine, centrifugalized in the usual manner, fails, it may occasionally succeed if as large a quantity of urine as possible is allowed to stand about twelve hours in a conical glass (containing a small piece of thymol), and the lowest portion withdrawn and centrifugalized. If no precipitate is formed by sedimentation, as large a quantity of urine as possible is centrifugalized in one and the same tube.

Cultural methods usually fail in the examination of the urine for tubercle bacilli, since their cultivation, even upon *Hesse's* agar, succeeds only when they are present in great numbers.

Animal inoculation yields more certain results, since it may be positive even when no tubercle bacilli can be detected in the stained smears. In the examination of the urine for tubercle bacilli the frequent presence of smegma bacilli must be borne in mind. Both belong to the group of acid-fast bacilli, and cannot, therefore, be distinguished from one another in stained smears. Neither do the staining methods, suggested by *Czaplewski*, *Pappenheim*, and others (cf. p. 331), by which only the tubercle bacilli are stained red, while the smegma bacilli are stained blue, allow a differential diagnosis to be made with certainty. These methods are, of course, especially inadaptable when only isolated, suspicious-looking bacilli are detected in the smears. Cultural methods are also of no service in differential diagnosis, since the cultivation of smegma bacilli is as yet impossible, and the cultivation of tubercle bacilli from the urine does not always succeed. Animal inoculation alone can furnish the proper means of differentiation, since smegma bacilli are not infectious for guinea-pigs. Animal inoculation should, therefore, be used in every case in which acid-fast bacilli are detected in urine obtained in accordance with the above-mentioned precautions.

Procedure of the Animal Test

For the animal test, for which guinea-pigs (half grown, about 250 gr in weight) are taken, the thoroughly centrifugalized urinary sediment is used after treating same in physiological salt solution or in bouillon. The inoculation is made subcutaneously in the inguinal region after shaving and cleansing with alcohol. The preference is given to the subcutaneous injection against the intra-peritoneal, because with other pathogenic germs present the animals often die after the intra-peritoneal injection much sooner from peritonitis and sepsis. Furthermore, the onset of the disease after subcutaneous inoculation can be observed, because it always starts at first with a localized tuberculosis on the point of inoculation, which also goes to show, that the infection was brought on by the injected material.

If the test proves to be positive, a swelling of the regional glands (popliteal glands) sets in during the second or the beginning of the third week; sometimes also an infiltration is forming on the point of inoculation, afterward the tuberculosis invades the inner organs. If these enlarged glands are removed—provided they are tubercular—smears taken from the glandular juice will show the tubercle-bacilli, and the diagnosis of tuberculosis can be made as early as in the second or third week after the inoculation. It is not necessary to make sections in order to find the tubercle bacilli. The extirpation of the glands is borne well by the animals without influencing the progress of the tuberculosis. *Bloch* has recommended to squeeze the inguinal lymph-glands before inoculating subcutaneously, in order to accelerate the tubercular changes by this mechanical insult as follows: take the inguinal fold between thumb and index-finger and rub the inguinal region repeatedly, always with the two fingers, going from underneath up to the surface, thus the inguinal glands are felt between the rubbing fingers as small nodules, and are squeezed by firm pressing. If the test proves to be positive, a node of about bean-size is found in the inguinal region ten to fourteen days after the inoculation; if extirpated, it shows a number of enlarged lymphatic glands in inflammatory infiltrated tissue. In the smears taken from the glandular juice numerous tubercle bacilli are found.

The pressing of the glands is to be omitted when numerous bacteria are found by the microscope, because the guinea-pigs, when squeezed, die sooner from the infection of these bacteria, while the animals, when not squeezed, survive this infection. Furthermore, it is to be noted, that not seldom, even two to three days after the inoculation, these squeezed and pressed glands swell without an existing tuberculosis, but they do not get larger in the course of the following days; sometimes they become smaller; sometimes abscesses are forming, in which case the pus is to be examined for tubercle bacilli. At any rate, these glands must not be extirpated too soon, it is best to inoculate two animals, but to squeeze the glands in one only.

If the animals are killed four to six weeks after the infection, in dissecting, the inguinal region is found to be infiltrated or ulcerating, the popliteal glands markedly swollen and a cheesy mass in the centre; numerous miliary nodules in the spleen, which is enlarged two to three times its size, and in the liver; the peritoneal and bronchial glands are enlarged with a cheesy mass in the centre; in the lungs also often numerous gray nodules are visible. To corroborate the diagnosis it is essential to find the tubercle-bacilli in the morbid products. In the tubercular nodules, which are rubbed between two cover-glasses for the purpose of examination, always only very few isolated bacilli are found.

If there is no swelling of the glands in the test-animals, they have to be watched for from six to eight weeks and must then be killed and dissected.

Typhoid Bacilli.—Cystitis and bacteriuria, caused by typhoid bacilli, have been frequently described in recent years. The bacilli may be observed in the urine as early as the end of the second or the beginning of the third week of the disease, but, as a rule, appear later, and often not until convalescence. The urine is acid in reaction, and contains an enormous quantity of typhoid bacilli. These are, as a rule, the only bacteria present.

On examining the sediment in a hanging-drop, numerous highly motile bacilli are seen. In stained smears they appear as small rods, which are decolorized by *Gram*. They are cultivated and identified according to the method described under Examination of the Fæces.

Gonococci.—Pure gonorrhœal cystitis is very rare. Cystitis following gonorrhœa is usually due to mixed infection. The diagnosis of gonorrhœal cystitis is especially difficult, since, even when gonococci appear in the urine in great quantities, the possibility that pus from the posterior urethra has become mixed with the contents of the bladder cannot be excluded. Concerning the detection of gonococci, cf. Examination of the Urethral Secretion.

Proteus Vulgaris.—In cystitis excited by the *Proteus vulgaris* the urine is ammoniacal in character. *Proteus vulgaris* appears alone, or with other micro-organisms, especially *Bacterium coli.*

Microscopical examination reveals rods of varying size, which frequently form long spiral threads, and for the most part are decolorized by *Gram;* occasional bacilli, however, if deeply stained, are not decolorized. In hanging-drops they appear highly motile. Their growth on gelatine is characteristic. Delicate, gray colonies develop, which soon sink into the gelatine and produce wavy excavations with a whitish mass in the centre, surrounded by a clear area. The colonies spread over the culture media by forming radiating branches, which may separate entirely from the mother-colony. *Proteus vulgaris* ferments grape- and cane-sugar, but not milk-sugar, and forms a large amount of indol. It decomposes albuminoid substances with the formation of foul-smelling products.

Bacillus pyocyaneus has been found both as an independent exciter of disease, and with other bacteria, in cystitis. (Concerning its microscopical and cultural characteristics, cf. p. 53.)

CHAPTER VIII

EXAMINATION OF THE URETHRAL AND PROSTATIC SECRETIONS

Bacteriological examination of the urethral secretion is directed principally toward the detection of gonococci, which are in the vast majority of cases the exciting cause of urethritis. Non-gonorrhœal urethritis is rare. It is usually excited by *Bacterium coli*, but staphylococci, pseudo-diphtheria bacilli, and micro-organisms of the normal urethral flora, may excite it.

In acute urethritis in the male, the secretion is taken from the urethra by means of a platinum wire, and spread in a thin, even layer upon a cover-glass or slide. In women the secretion of the urethra, or that of the cervix uteri, is used for examination. Vaginal secretion is absolutely unfit for use, since it usually contains a large number of different micro-organisms, among which the gonococci can scarcely be detected. In young girls, however, the detection of gonococci in the vaginal secretion is very easy.

In chronic gonorrhœa in the male, "the morning drop," or the filaments which appear in the urine, are examined. The latter are most numerous in the first morning urine. Since they are washed from the urethra with the first stream of urine, and are easily dissolved in a large quantity, only a small quantity (about the first 20 or 30 cc) is collected for examination. The filaments are removed with a pipette, and carefully spread upon a cover-glass or slide.

Microscopical Examination.—Smears are stained according to *Gram*, and with a very dilute methylene-blue solution (Plate XII, Fig. T), which only slightly stains the nucleus, but stains the cocci intensely. The numerous double-staining methods which have been suggested have no diagnostic value, though they simplify the detection of isolated cocci. The method of *May* and *Gruenwald* is to be recommended. The methods of *Schaeffer*, *Pick*, and *Jakobsohn*, and *Krystallowicz's* modification of *Pappenheim's* method, should be mentioned. With the latter, the gonococci are stained brilliant red, the nuclei pale green, and the protoplasm faintly pink (cf. p. 334). These methods afford good results only when the smears are thinly and evenly spread. The gonococci appear in stained smears as diplococci, which are usually biscuit- or coffee-bean-shaped. They rarely lie singly, but usually in groups. In purulent secretion they lie almost exclusively within the pus-corpuscles, which often appear stuffed with them. In the first stage of gonorrhœa, in which the mucous secretion contains numerous epithelial cells and fewer leucocytes, the gonococci frequently lie outside of the cells, often almost completely covering them. They also lie for the most part outside of the cells in the mucopurulent secretion of chronic gonorrhœa. The fact that they are decolorized by *Gram* is of value in differential diagnosis.

Cultural Procedures.—Gonococci do not grow upon the usual culture media. A culture medium containing serum (human serum is best) is necessary for their cultivation. The medium must be prepared in such a manner that the albumin contained in the serum is not coagulated.

Wertheim's serum-agar, which consists of a mixture of 2 to 3 parts nutrient-agar with 1 part human blood-serum, is the most favorable medium upon which to cultivate them.

In place of blood-serum other human serous fluids, as hydrocele-cystic, ascites-, and hydrothorax-fluids may be used. The latter media are, however, not absolutely reliable, since, for reasons unknown, the gonococci occasionally fail to develop. The serum is never mixed with the agar until shortly before use, when it is heated to 40° C., and poured into the agar, which has been melted and cooled to 50° C.; the medium is allowed to solidify in obliquely placed tubes. *Wassermann's* swine-serum nutrose-agar should also be mentioned, upon which, however, the gonococci develop irregularly and sparingly. Concerning the preparation of the culture media, cf. pp. 354, 355.

Appearance of the Cultures.—After twenty-four hours' growth at 36° C., round, slightly gray, transparent colonies, of characteristic mucoid consistency, and of about the size of a small pin's head, have developed. The individual colonies do not coalesce, and resemble those of streptococci. The numerous degeneration forms, which may be seen beside the typical diplococci in smears made even from twenty-four hours' cultures, are characteristic. The degeneration forms appear swollen, and stain poorly.

Differential Diagnosis.—In examining secretions from the genital organs, the morphological and staining characteristics furnish sufficient evidence upon which to make a certain diagnosis. The peculiar form of the gonococci, their characteristic position, and the fact that they decolorize by *Gram*, usually render it possible to differentiate them at once from other pyogenic cocci. Occasionally the detection of suspicious-looking diplococci may necessitate cultural procedures—namely, when the diagnosis is of great importance (marriage consent, medico-legal cases). In such cases, in addition to the serum cultures, smears are made upon ordinary agar, since the absence of growth upon the latter is of especial diagnostic value. In cases of chronic urethritis in which no gonococci are detected microscopically, cultural procedures usually fail also. Examination is therefore, as a rule, limited to thorough microscopical examination. This should be repeated as often as possible, and, if necessary, may be preceded by provocative irritation. Cultural methods are indispensable for the identification of gonococci in extra-genital diseases.

It is occasionally necessary to restain, according to *Gram*, a smear which contains suspicious-looking cocci (for example, in the examination of filaments). This is done by removing the Canada balsam and cedar oil with xylol, which is in its turn removed with absolute alcohol, washing with water, decolorizing with 3 per cent. hydrochloric acid alcohol, and again washing, after which the smear may be stained according to *Gram*.

Prostatic Secretion

Prostatic secretion is obtained for examination by massage of the prostate following irrigation of the anterior urethra. It is examined in the same manner as the urethral secretion.

CHAPTER IX

EXAMINATION OF THE BLOOD

I. Determination of the Specific Gravity

Hammerschlag's Method.—A mixture of chloroform (specific gravity, 1.527) and benzol (specific gravity, 0.880), in the ratio of 2 to 5.5, is placed in a dry 100 cc glass cylinder. The mixture should have a specific gravity between 1.050 and 1.055, and should fill the cylinder about three-quarters full. A medium-sized drop of blood is taken from the ball of the finger, or the lobe of the ear, into the mixture. During the introduction of the drop and the following manipulations, care must be taken that it is not broken up into smaller drops. If the drop of blood sinks to the bottom, the specific gravity of the mixture is lower than that of the blood, in which case a few drops of chloroform are added, and mixed with the fluid, by carefully tipping the cylinder, which is closed with the palm of the hand. If the drop now remains at the surface, a drop of benzol must be added, and the fluid again mixed. The addition of chloroform or benzol is continued until the drop assumes a fixed position in the fluid, neither rising nor sinking. The benzol-chloroform mixture and the drop of blood are then of the same specific gravity. The specific gravity of the mixture is determined by means of an areometer. The benzol-chloroform mixture may be filtered through a dry filter and used for subsequent examinations. The specific gravity of normal blood is 1.055 to 1.060.

II. Determination of the Freezing-Point

The determination of the freezing-point is most conveniently carried out with the blood-serum. Since at least 10 cc of serum are necessary for each determination, the blood must be obtained by venesection or cupping. The freezing-point is determined in the same manner as that of the urine (cf. p. 137).

III. Estimation of Haemoglobin

Hæmoglobin may be estimated either by estimating the intensity of the color of the blood or the iron contained in it. Since a large quantity of blood is necessary for the exact estimation of iron, and since such an estimation consumes considerable time, only the methods for estimating the intensity of the color of the blood are used for clinical and practical purposes. These allow the rapid estimation of hæmoglobin with a very small quantity of blood. Of the various forms of apparatus which have been suggested for the estimation of hæmoglobin, the two following can be recommended for clinical use:

1. *Sahli's Modification of Gowers' Hæmoglobinometer.* —The instrument consists of two glass tubes of exactly equal diameter, one of which is three-quarters full of a solution of hæmatin chloride and closed at both ends. The second is closed at but one end, carries a scale with divisions from 10 to 120, and receives the blood to be examined. Both tubes are set in a black frame which has a white background, so that differences in color can be easily recognized. In addition, a capillary pipette for measuring 20 cubic millimetres, a dropper for diluting the blood, and a bottle for dilute hydrochloric acid, are

furnished with the apparatus. The hæmoglobin is estimated as follows:

A few drops (to the mark ten or twenty) of a dilute (0.2 per cent.) hydrochloric acid solution are placed in the graduated tube. The capillary pipette is now filled with blood to the mark by suction, and the blood quickly blown out upon the bottom of the tube containing hydrochloric acid. The tube is thoroughly shaken, whereupon the color of the blood is altered, owing to the formation of hæmatin chloride. The solution becomes dark brown, and upon dilution with distilled water assumes the color of the test fluid. The dilution with distilled water must be made carefully, a drop at a time. The level of the liquid in the tube shows on the scale the quantity of hæmoglobin present. One hundred on the scale represents the normal quantity of hæmoglobin. This estimation is best performed by daylight, and yields results thoroughly useful for practical purposes.

2. *Tallquist's Hæmoglobin Scale.*—This is an empirical scale of colors, which represent the shades of blood-red corresponding to definite percentages of hæmoglobin. A book of filter-paper is furnished with the scale. The estimation of hæmoglobin with this scale is very simple; a drop of blood from the ball of the finger, or the lobe of the ear, is absorbed with a leaf of the filter-paper. The color of the drop is then compared with the scale; the number opposite the shade which most nearly corresponds to it gives the percentage of hæmoglobin. This method is not very accurate (errors of 10 to 20 per cent.), but is very simple, and quickly performed. *Grawitz* recommends that the blood-spot be cut out with scissors and placed upon the color scale directly, since the colors can thus be more accurately compared.

IV. Enumeration of Blood-Corpuscles

The red and white blood-corpuscles are counted with the *Thoma-Zeiss* hæmocytometer. This consists of two mixing-pipettes and a counting chamber. The mixing-pipettes are capillary tubes, about 10 centimetres in length, and with a bulb in their upper half, which contains a freely movable glass bead; 0.5 and 1 are marked on the capillary tubes below the bulb, and 101 and 11 respectively above it. The mixing pipette with the mark 101 is used for counting the red, that with the mark 11 for counting the white corpuscles.

The counting chamber consists of a slide, upon which a glass frame with a circular opening is cemented. In the centre of the opening is a round glass plate, upon the surface of which a network of large and small squares is marked. The frame extends exactly 0.1 millimetre above the surface of this glass plate, so that if a cover-glass is placed upon the frame, its under surface is exactly 0.1 millimetre above the surface of the glass plate.

The red blood-corpuscles are counted in the following manner: A drop of blood is drawn up into the proper pipette to the mark 0.5 the excess of blood removed with the tip of the finger, and a 2 per cent. solution of sodium chloride at once drawn up to the mark 101. Care must be taken that no air enters the pipette. The pipette is shaken to obtain even dilution of the blood in the bulb. Three or four drops are now blown from the pipette, and a medium-sized drop placed upon the glass plate of the counting chamber. The drop is covered with a cover-glass, care being taken that no bubble of air is formed. The cover-glass must be in such close contact with the frame that *Newton's* rings are seen. The counting chamber is then placed with its centre under the microscope (magnification of about 180 to 220), so that the network of lines and the red blood-corpuscles lying upon it are clearly seen.

The network of the *Thoma-Zeiss* apparatus consists of sixteen large triple-contoured squares. Each large square is divided by single lines into sixteen small squares. In counting the red blood-corpuscles, it is advisable to centre a large square, and to count and note the corpuscles in each small square, counting only the cells lying within the squares and upon their upper and left borders. Five large squares ($=5 \times 16=80$ small squares) are counted. The number of corpuscles is calculated as follows:

The side of each small square is $\frac{1}{20}$ millimetre; its surface is, therefore, $\frac{1}{20} \times \frac{1}{20} = \frac{1}{400}$ of a square millimetre. Since the thickness of the blood layer is $\frac{1}{10}$ millimetre, the volume of blood contained in one small square = $\frac{1}{400} \times \frac{1}{10} = \frac{1}{4000}$ of a cubic millimetre.

The number of blood-corpuscles in a cubic millimetre of blood is calculated from the formula

$$x=\frac{m.\ n.\ 4{,}000}{q},$$

in which m = the number of red blood-corpuscles counted, n = the dilution of the blood, and q = the number of small squares counted.

The enumeration of the leucocytes differs from the above as follows:

1. Instead of the mixing-pipette with the mark 101, that with the mark 11 is used (this allows a dilution of 1:10 or 1:20).

2. A dilute solution of acetic acid (0.3 to 0.5 per cent.) is used as diluent, in which the red blood-corpuscles are dissolved, and the white therefore easily recognized and counted.

3. Because of the small number of leucocytes contained in a field, a large number of squares must be counted. For this purpose *Turk's* modification of the *Thoma-Zeiss* counting chamber is best used. It has, in addition to the sixteen large squares, a number of squares of the same size, but which are not divided into smaller squares. This renders it possible to count a much larger number of leucocytes. At least 100 to 150 leucocytes should be counted in each enumeration.

We note the number of leucocytes in each square. We count in the same way as we count the red cells. *Illustration:* 130 leucocytes were counted in 40 large squares. The number of leucocytes in one cubic mm then are

$$x = \frac{130.\ 4000.\ 10}{40.16}$$

(provided that the blood was diluted ten times).

A certain amount of practice and great care in carrying out the details are necessary, in order to obtain an accurate count of blood-corpuscles. The counting chamber and the mixing pipettes must be absolutely clean and dry. After each count the pipettes should be cleansed, first with a 1 per cent. solution of sodium hydrate, second with water, third with alcohol, and finally with ether.

V. Histological Examination

(*a*) Examination of Fresh Specimens

The cover-glasses and slides used for the histological examination of the blood must be absolutely clean, and must have been washed with alcohol and ether. The under surface of a cover-glass is touched to a drop of blood, as it issues from a prick of the finger or ear, and placed on a slide, without pressure, and without allowing it to shift after it has touched the slide. The blood spreads spontaneously in a very thin layer. If the specimen has been properly prepared, the cells lie in the centre detached from one another, and rouleaux formation is seen only at the periphery. In the examination of the fresh specimen the following points should be noted:

1. The intensity of the color of the red blood-corpuscles and their rouleaux formation.

2. Morphological alterations in the red blood-corpuscles (poikilocytosis, presence of nucleated red blood-corpuscles).

3. Increase in the number and alteration of the structure of the leucocytes.

4. Presence of micro-organisms (spirilla of relapsing fever, plasmodia of malaria).

(*b*) Examination of Stained Specimens

1. ***Preparation of Smears.***—A thin cover-glass (0.1 in thickness) is held at one edge in a pair of *Ehrlich's* blood-forceps, and another cover-glass is held in a pair of ordinary forceps, and the centre of its under surface touched to a small drop of blood, as it issues from the finger or ear. The cover-glass with the drop of blood is then quickly placed upon the other, without pressure, whereupon the blood spreads in a capillary layer. The upper cover-glass is now seized by the edge with the thumb and forefinger of the right hand, and drawn from the lower. The cover-glasses are then set aside with the smeared surfaces up. Spectral colors are seen at the best spread portions of the specimens when the glass is viewed at an acute angle.

2. ***Fixation.***—The best fixing fluids for blood specimens are absolute alcohol, alcohol and ether, and formalin.

Specimens are fixed in alcohol and in alcohol and ether āā, for five minutes to twenty-four hours. Formalin fixes in two to three minutes. The best specimens are, however, obtained by fixation with heat, which, according to *Ehrlich's* original instructions, is carried out with a hot copper plate, at a temperature of 100° to 130° C. Special fixing-ovens have been constructed according to *Ehrlich's* suggestions. Since, however, fixation upon *Ehrlich's* copper plate and in the special ovens consumes considerable time, and is therefore unsuited for the daily use of the practising physician, *Kowarsky*[1] has shortened the procedure considerably. The smears are placed with the blood up, upon a hollow copper cylinder (Fig. 38). A crystal of urea is placed in the hollow in the upper surface of the cylinder. The cylinder is heated just above the flame of a *Bunsen* burner, or alcohol lamp, until the crystal of urea begins to melt. This takes place between 132° to 135° C. The cylinder and smears are set aside until they have cooled. The entire fixation takes but two to three minutes.

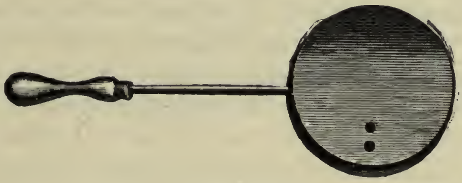

FIG. 38.

3. **Staining.**—I. *Ehrlich's Triple Stain.*—*Ehrlich's* triacid stain has the following composition:

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[1] *Dr. A. Kowarsky, Berliner klin. Wochenschr.,* 1903, No. 10.

Sat. watery solution of orange-G . . .	13.0 to 14.0
Sat. watery solution of acid fuchsin . .	6.0 to 7.0
Aqua destillata	15.0
Alcohol	15.0
Sat. watery solution of methyl-green . .	12.5
Alcohol	10.0
Glycerine	10.0

The ingredients are measured, and placed in the same graduated measure in the above order and thoroughly shaken. It is advisable to filter the stain before using. Specimens are stained five to ten minutes.

II. *Giemsa's Method of Staining.*—The azure-eosin stain of *Giemsa* can be purchased as "*Giemsa* solution" (new), and must be freshly diluted for the staining of blood, so that one drop of this solution is added for each drop of distilled water. The specimen is first fixed in methyl alcohol for five minutes and then stained in the diluted stain for fifteen to twenty minutes. The *Giemsa* solution shows up especially the chromatin of the nuclei; also parasites of the blood.

III. *Simultaneous Staining and Fixation According to May and Gruenwald.*—The staining principle of the *May* and *Gruenwald* dye is a chemical combination of eosin and methylene blue. If a 0.1 per cent. watery solution of eosin and a 0.1 per cent. solution of methylene blue are mixed, and allowed to stand for some time, a new dye is precipitated. This is collected on a filter, and washed with cold water until the water runs away nearly colorless. A saturated solution of the dye so isolated is made with methyl alcohol. This is placed in a wide-mouthed glass, and used for staining and fixing blood specimens. The freshly spread and air-dried smear is held with forceps in the stain for two minutes. It is then rinsed in a beaker of water, which contains a few drops of the stain, until it assumes a pinkish-red color.

IV. *Leishman's Method of Staining and Simultaneous Fixation.*—*Leishman's* stain can be purchased ready for use. Five to ten drops of the stain are put on the dry specimen; after half a minute double the number of drops of aq. dest. are added, and the water and stain are mixed carefully by means of the cover-glass. This staining mixture remains for five minutes on the specimen, after which it is washed off with water. A few drops of water are left on the specimen for one to two minutes, until the water is colored a light green. The specimen is then washed, dried between filter-paper, and then proceeded with in the usual manner. This staining method gives excellent results and is to be recommended in the daily practice.

(c) Sketch of the Morphology of the Blood

NORMAL BLOOD-CORPUSCLES

1. ***Normal Red Blood-Corpuscles (Normocytes).***—These are biconcave discs which have no nuclei, and are composed of a stroma containing hæmoglobin. Their average diameter is 7 to 7.5 μ. With *Ehrlich's* triacid stain, normal erythrocytes are orange in color, if the specimens have been properly fixed (at 135° C.). If the specimens have been fixed at a lower temperature the corpuscles are redder. With ordinary eosin-methylene blue they are red; with *May* and *Gruenwald's* stain they are pinkish-red. In fresh specimens they are distinctly yellow.

2. ***Lymphocytes.***—These are cells about the size of red blood-corpuscles, or somewhat larger, with narrow, homo-

geneous (non-granular) protoplasm and a spherical nucleus, which nearly fills the entire cell. With the triacid stain, the nuclei appear greenish- to blackish-blue, the protoplasm pink; with eosin and methylene blue the nucleus and protoplasm are stained blue. The lymphocytes constitute about one-fourth of all the leucocytes normally present in the blood.

3. **Large Lymphocytes.**—These differ from the ordinary lymphocytes only in size. They appear in the blood of young children, normally, up to about 10 per cent., but are rare in healthy adults. They are, however, commonly found in acute and lymphatic leukæmia.

4. **Polynuclear Neutrophilic Leucocytes.**—These are twice the size of lymphocytes and have several nuclei, or one polymorphous nucleus. They constitute the majority (about 75 per cent.) of the normal leucocytes. The protoplasm is distinctly granular. The granules are usually very small. With the triacid stain, the nucleus stains greenish to deep blue, the granules violet, the protoplasm between the granules pink. With eosin and methylene blue (first eosin, then methylene blue) the nucleus appears blue, the granules red, while the protoplasm remains colorless.

5. **Acidophilic or Eosinophilic Leucocytes.**—These are easy to recognize, even in unstained specimens, by the coarse, highly refractive, round granules in their protoplasm. As the name implies, the granules stain with any acid stain, and are, therefore, deep red with triacid and eosin-methylene blue, as well as when stained according to *May* and *Gruenwald.* Two varieties of eosinophilic cells are distinguished:

(*a*) *Polynuclear or Normal Eosinophiles.*—These constitute ordinarily 2 to 4 per cent. of the total leucocytes of the blood. They have two or three nuclei, which do not stain as intensely as those of the neutrophiles.

(*b*) *Mononuclear Eosinophiles.*—These appear in the blood only under pathological conditions.

6. **Basophilic Leucocytes or Mast-Cells.**—The protoplasm of these cells contains coarse granules about the size of eosinophilic granules, which are, however, not always so round nor of the same shape. These granules have a marked affinity for basic stains (methylene blue), and stain, therefore, dark blue with eosin-methylene blue and with the *May-Gruenwald* stain. They are colorless in specimens stained with the triacid mixture.

Mast-cells are mono- or polynuclear, and about the size of the neutrophiles, though sometimes smaller. They appear in very small quantity in normal blood (0.5 per cent.). They are most easily detected with the *May-Gruenwald* stain.

7. **Transition Forms.**—Their size corresponds mostly with the neutrophiles. The protoplasma is mostly basophile and shows a different behavior; it appears homogeneous with the triacid stain. In the *Giemsa* and *Leishman* specimens are sometimes seen granulations, which at times show the characteristics of neutrophile granules and at times the characteristics of the azurophile granules of lymphocytes. The nucleus is mostly semicircular or of horse-shoe shape.

8. **Blood Platelets.**—These are small (2 to 3 μ in diameter), quadrilateral or round colorless objects, which frequently lie in groups, and are present in great quantity in normal blood. They come, in all probability, from the red blood-corpuscles, and are considered to be products of the decomposed nuclear substance. They are basophilic, and stain faintly blue with eosin-methylene blue, and faintly pink with the triacid mixture.

ABNORMAL AND PATHOLOGICAL BLOOD-CORPUSCLES.

(*a*) *Abnormal and Pathological Red Blood-Corpuscles* (Plate XIII, Fig. U).

1. **Poikilocytes.**—These are not circular, but are pear-, spindle-, dumb-bell-, or kidney-shaped. They are considered to be fragments of normal erythrocytes, and appear in the blood in anæmic conditions.

2. **Macro- and Microcytes.**—The first are larger than the normal erythrocytes, and frequently have no concavity. They appear usually in severe anæmia. The microcytes are smaller than the normal erythrocytes, and are usually found in the blood, together with poikilocytes.

3. **Nucleated Red Blood-Corpuscles of Normal Size (Normoblasts).**—The nucleus is spherical, usually excentrically placed, and stains very intensely (deep blue).

4. **Large Nucleated Red Blood-Corpuscles (Megaloblasts).**—These are about the size of the macrocytes, and frequently have two nuclei. The nucleated erythrocytes (both forms) appear only in severe forms of anæmia.

5. **Erythrocytes with Basophilic Granulation.**—These erythrocytes have granules of varying size (from a grain of dust to one-fourth the size of the nucleus) in their protoplasm, which stain well with all basic stains, and, therefore, blue with all staining methods. They are considered by most authors as the remains of nuclei, and appear in severe forms of anæmia (anæmia following lead-poisoning).

6. **Polychromatophilic Erythrocytes.**—Erythrocytes which have lost their normal affinity for acid dyes to a greater or less extent, and have assumed a slight affinity for basic dyes are designated as polychromatophilic. They stain a bluish-red or violet, instead of pinkish-red, with eosin-

methylene blue. They do not appear in normal blood, but are frequently seen in various forms of anæmia.

(*b*) *Pathological Leucocytes.*

1. ***Myelocytes, or Mononuclear Neutrophiles.***—The spherical nucleus occupies the greater part of the cell, and usually stains faintly—much more faintly than the nuclei of the polynuclear leucocytes. The neutrophilic granules of the protoplasm are, as a rule, comparatively faintly stained. These cells constitute the majority of the cells of bone-marrow. They appear in great numbers in the blood in myelogenic leukæmia, and occasionally in severe forms of anæmia in children. They are considered to be the antecedents of the normal polynuclear neutrophiles.

2. ***Eosinophilic Marrow-Cells.***—These are mononuclear eosinophiles. They are usually larger than the polynuclear eosinophiles, and have a somewhat smaller nucleus than the myelocytes. They are seen, together with the latter, in leukæmia.

3. ***Non-Granular Marrow-Cells.***—These are very large, delicate cells with homogeneous protoplasm. The nucleus and protoplasm stain very faintly. The protoplasm is faintly basophilic. Occasionally a suggestion of neutrophilic granulation is seen in the protoplasm, so that these leucocytes may be considered as transitional forms of myelocytes. They appear in the blood in myelogenic leukæmia.

VI. Bacteriological Examination of the Blood

1. Examination of the Blood in Stained Smears

The blood is examined in stained smears in the diagnosis of malaria and relapsing fever.

(*a*) ***Malaria.***—The examination of the blood for the malarial parasite is best made with blood obtained during the decline of the fever, or directly after it. The patient should have taken no quinine for several days before the examination.

The blood is obtained by pricking the finger or lobe of the ear with a sterile needle. The edge of an absolutely clean cover-glass is touched to a drop of blood as it issues, and is placed on a second cover-glass, or slide, so that the right-hand angle made by cover-glass and slide is about 45°. The blood spreads along the cover-glass, and the latter is carried across the slide from right to left. In this manner the blood is spread in a thin layer, without pressure. After the specimen has dried in the air it is fixed for three minutes in alcohol and ether āā.

The staining is done either after the method of *Manson* or of *Giemsa.*

Manson's Method.—Borax-methylene blue (cf. p. 329), which is used as stock solution, must be diluted with aq. dest. until the resulting staining solution just looks transparent in the test-tube. Into this diluted staining solution the specimen is immersed for five to ten seconds, washed in a glass of plain water until the color is of a dull green, then dried between filter-paper and examined with oil immersion. The orthochromatically stained red cells appear green, the metachromatically stained are gray blue, the nuclei of the white cells and the parasites are blue. The blood placques are of a dull gray blue, whose margins are blurred in contradistinction to the parasites whose margins are sharply defined. The pigment of the parasites varies from yellow to dark brown.

Giemsa's Method.—The commercial *Giemsa* solution is diluted with aq. dest. in the proportion of one drop of the stain to 1 cc of water under slight agitation in the beaker. The specimen is put into a small dish, the specimen being turned to the bottom, the diluted staining solution is poured over it and the specimen is left in this stain for one to two hours. The parasites appear blue with a refracting red chromatin nucleus, the red cells are red and the nuclei of the leucocytes are of lilac or dark violet color.

Malarial organisms are protozoa which have a double cycle of existence—an asexual, as parasite in human erythrocytes, and a sexual, in the mosquito (anopheles). They are divided into two groups: Large parasites, to which the parasites of tertian and quartan fever belong, and the small, annular parasites of tropical fever (æstivo-autumnal fever). The types of fever belonging to the different forms of malaria depend upon the cycle of development of the parasites: The parasite of tertian fever requires forty-eight hours for its development, that of quartan fever seventy-two hours, and that of tropical fever twenty-four to forty-eight hours. The infected person is free from fever as long as the parasites are developing. The fever begins only after they have completed their development, simultaneously with the appearance of the so-called division forms. Quotidian fever is not caused by a particular parasite, but is due either to a double tertian or a triple quartan infection.

The Tertian Parasite (Plate XIII, Fig. V, and Plate XIV, Fig. W).—If the blood is obtained during the height of the fever or during its fall, and stained according to *Manson*, the youngest parasites are seen lying upon the green erythrocytes in the shape of small, blue, ovoid objects, which appear to be distinctly annular, as well as small bluish rings, which have on one side a crescentic thickening, and on the opposite a slight knob (small tertian parasite, seal-ring form). Twenty-four hours later the parasites are found to have grown considerably: they are about twice the size of the small tertian rings, and some still show a distinct annular form (large tertian rings), while others (large parasites) have lost the annular form and appear as blue, round, or irregular discs. Both the large tertian rings and the large parasites contain yellow or blackish-brown pigment. The erythrocytes, attacked by the parasites, are enlarged and pale. At first the pigment is irregularly distributed throughout the protoplasm of the parasites. A few hours before the next paroxysm it is collected at the centre of the parasite, and the parasite itself shows a distinct differentiation (division form). Finally, it divides into fifteen to twenty-five small, round, or oval blue bodies (spores), from which the new parasites develop.

In addition to these asexual forms, those which in the mosquito serve for the sexual development of the parasite (gametes) are seen. These resemble the large parasites in size and form, and either lie free, or nearly fill the pale, enlarged erythrocytes. They differ from the asexual forms as follows: They stain less intensely at the border (pale grayish-blue or grayish-green), or they have an absolutely unstained area in the centre; further, they show no differentiation in their protoplasm, throughout which the pigment is irregularly distributed. Male and female gametes, which show differences in staining and pigmentation, are distinguished in stained smears.

The Quartan Parasite.—The quartan parasite cannot be distinguished in the first stage of its development from the tertian. In specimens obtained during the decline of the fever, rings are seen which exactly resemble the small tertian rings. From these the band forms, characteristic of the quartan parasite, are developed. The erythrocytes attacked by the parasites, in contradistinction to those in tertian fever, are neither bleached nor enlarged, and are traversed by blue, heavily pigmented bands, which gradually enlarge, become quadrilateral, and finally completely fill the corpuscle. The quartan parasite divides, after the pigment has collected at one point, into sixteen to twenty-four spores. In addition to the asexual forms, sexual forms are also seen, which resemble those of the tertian parasite.

The Tropical Parasite (Plate XIV, Fig. X).—During the rise of the fever, especially in the first attack, no parasites are found, or at most only occasional blue rings, which are much smaller than the small tertian rings, and have no crescentic thickening at their periphery (small tropical rings). Their further development, in contrast to the other forms of malaria, does not take place in the circulating blood, but in the inner organs (spleen, brain, bone-marrow), so that division forms are not seen in specimens made from the blood. The appearance of the gametes, which are crescentic, is typical of the tropical parasite. They stain more intensely at the poles than in the centre, at which the pigment is arranged in the form of a wreath.

The Differential Diagnosis between the different forms of malaria can be made with certainty only when careful measurement of the temperature accompanies the microscopical examination. In doubtful cases the temperature must be taken every three hours, day and night. The tertian parasite is characterized by the large pigmented rings and by the enlargement of the erythrocytes; the quartan by the band forms and the absence of enlargement of the erythrocytes; the tropical by the crescents. If the parasites are found in the microscopical specimen only in the form of small rings, it is impossible to make a diagnosis from the microscopical examination alone, since the small tertian and quartan rings have the same appearance, and resemble the large tropical rings. Further, the possibility of mixed infection must be remembered, as well as the alteration in the appearance of the different forms, due to the ingestion of quinine before the paroxysm (quinine forms).

(*b*) ***Spirilla of Relapsing Fever*** (Plate XV, Fig. Y).—For the detection of spirilla of relapsing fever the blood must be obtained during the fever, since, as a rule, the spirilla appear in the circulating blood but a few hours before the rise of the fever.

The blood is collected and the smears prepared in the same manner as for the detection of malarial parasites.

The staining is done with the diluted *Giemsa* stain (one drop to one cc of distilled water). The examination of the fresh specimen is best done with dark illumination.

The spirilla, discovered by *Obermeyer*, are highly motile, very fine spiral threads, with pointed ends, 10 to 40 μ in length and 1 μ in thickness. They usually lie singly or a few side by side, and rarely form snarls.

In order to examine the blood for trypanasomes the specimen is stained in the same way as for the examination for malaria.

2. The Examination of the Blood by Means of Culture Media

The most important bacteria, whose presence in the blood is demonstrated by means of culture media are: strepto-, staphylo-, pneumo-, and gonococci; typhus-, paratyphus-, coli-, pyocyaneus-, plague- and anthrax bacilli. For the purpose of making cultures it is best to take the blood from the median vein by puncture.

The arm hanging down at the side of the body is tied with a bandage or something similar above the elbow, the elbow region over the median vein is first thoroughly washed with soap then with alcohol, ether, and sublimate and the sublimate is removed by washing with freshly boiled water. The freshly boiled cannula of the syringe is plunged into the vein, which stands out prominently, and the blood is aspirated. The bandage is removed from the arm before the syringe is withdrawn.

The blood may also be removed by means of a sterile cup or by puncturing the finger or the ear. The latter method has the drawback, that, as a rule, the blood becomes contaminated by skin cocci and that we cannot always get a sufficient quantity. The blood is permitted to drop directly on the culture medium or we can carry it to the culture medium with a sterile pipette.

Usually 3 to 5 cc of blood are obtained, which is at once put into a small flask with 50 to 100 cc of bouillon, or is mixed with melted agar which has cooled down to 45° and which is immediately poured over plates. *Cannon* recommends to squirt the blood into glasses of slanting agar upon which it coagulates after it has been equably distributed by proper motions and after the glass has been put in a slanting position. The cultures are put into the incubator for twenty-four hours and then examined. Then smears are made on agar plates from the bouillon.

We have to be especially careful when we find staphylococci, as even after the most careful operation the cocci may have come from the skin. Such possibility is especially to be considered, when the cultures of the blood show staphylococci, while different germs are found in the pus. In order to determine whether we are dealing with pathogenic staphylococci we can make the agglutination test and examine for toxine formation.

Blood cultures of the typhoid and paratyphoid bacilli are made in the media of *Conradi* and *Kayser* to which bile has been added. *Conradi's* medium consists of fresh bovine bile, to which is added 10 per cent. peptone and 10 per cent. glycerine; of this 5 cc are put into test-tubes and the test-tubes are sterilized in live steam for two hours. *Kayser* uses merely the gall alone, without any further addition. These sterilized tubes must be kept cool. About 2.5 cc of blood are put into such a test-tube. However, even smaller quantities of blood may give positive results. These gall media enable us also to make cultures from the coagulated blood for the typhoid and paratyphoid bacilli, and we can use for such purpose the blood-cakes of blood specimens which have been used for making the *Gruber-Vidal* test. The blood-cake is transferred directly into the gall medium of the test-tube after pouring off the serum. By such cultures we are able to make an early diagnosis at a time when the agglutination test is negative.

The test-tubes with gall media to which the blood has been added, are put into the incubator for fourteen to twenty hours. Without shaking the test-tubes a few loops are carefully removed from the surface of the gall media, and after this a larger quantity, about 0.5 cc, which are transplanted upon a large *Endo-* or *Conradi-Drigalski* plate, and rubbed into this with a glass spatula. The examination of these media is made in the usual manner.

The typhoid bacilli are found in the blood during the entire febrile stage, very often already in the first days of the disease and are especially numerous during the stage of the eruption of the roseolæ. During the afebrile stage they cannot be demonstrated. Likewise, after the febrile state is gone, an attempt to prove their presence is often fruitless. Even at the height of the fever we may not be successful if the disease is of a mild type.

A culture may be made not only from the circulating blood, but also from the roseolæ in which they are always found. The cultures are made in the following way after *Neufelds:* The skin is first washed with a mixture of even parts of alcohol and ether, a very slight incision is made with a sharp scalpel into the roseola, a little matter is scraped out with the scalpel and put at once into bouillon. The incision must be made so superficially that no blood should come, as the typhoid bacilli are in the tissue of the roseola and not in the blood. Should blood come then a little bouillon is dropped on the wound so as to dilute the blood immediately. This bouillon diluted blood is immediately transferred into the bouillon test-tubes. The test-tubes are the nput into the incubator which is kept at 37° C., for eight hours, after which time smears are made on agar, and the bacterial growth is examined the next day by means of agglutination, vaccination of litmus-whey, etc. In the bouillon test-tubes are found usually staphylococci besides the typhoid bacilli.

The newly appearing roseolas are best suited for examination, and by preference several of them ought to be examined at the same time. Several specimens are taken from each roseola and implanted into the bouillon test-tubes. Good results were obtained also by *Schmiedecke* who followed the method of *Neufelds* with the variation that he removed the skin over the roseolas in very fine layers and planted these in the bouillon.

Instead of using bouillon the test-tubes containing the gall media can also be used.

(3) EXAMINATION OF THE BLOOD BY MEANS OF ANIMAL INOCULATION

Animal inoculation is of special value for the detection of anthrax and plague bacilli in the circulating blood.

Anthrax Bacilli.—White mice or guinea-pigs are used as test-animals, and are inoculated subcutaneously with 0.2 to 0.3 or even 1.0 cc of the blood obtained by venepuncture. If the blood contains anthrax bacilli, the animals die of anthrax septicæmia, and the bacilli can be detected in the blood and viscera by microscopical and cultural examination.

Plague Bacilli.—Rats or guinea-pigs are inoculated with blood.

Animal inoculation may also be used for the detection of STREPTOCOCCI in the blood. It is not, however, as reliable as are cultural procedures, since streptococci which are highly virulent for man may be avirulent for animals, so that a negative result does not necessarily exclude the possibility of the presence of streptococci in the blood. White mice are used as test-animals, and are inoculated intraperitoneally with 0.5 to 1.5 cc of blood.

If the blood contains streptococci virulent for mice, the animals die of streptococci septicæmia.

(4) SERUM DIAGNOSIS.

Serum diagnosis depends upon the fact that specific reaction products—agglutinins and bacteriolysins—appear in the blood of persons who are suffering or have suffered from infectious diseases. This observation has found practical application principally in the diagnosis of typhoid fever. Shortly after *Grueber* had determined that the blood of patients convalescing from typhoid fever has the power to agglutinate typhoid bacilli, *Widal* called attention to the fact that the blood-serum contains the agglutinins even during the course of the disease, and often in its first stage.

Performance of the Agglutination Test (the Widal Reaction)

The blood is best obtained by venepuncture or cupping —about 2 cc are taken. If these methods are impracticable, the blood is obtained from a prick in the ball of the finger, and collected in a small centrifuge tube. After the blood has coagulated, the clot is loosened with a sterile platinum needle from the sides of the tube. In the course of the next few hours, during which the blood is kept in an ice-chest, sufficient serum is usually obtained. This is removed with a pipette, diluted ten times with a sterile 08.5 per cent. solution of sodium chloride (1 part serum and 9 parts salt solution), and centrifugalized until clear. From this further dilutions, in the ratios of 1:20, 1:40, 1:50, 1:60, etc., are made. In 1 cc of each dilution one loop of an eighteen to twenty-four hours' agar typhoid culture, whose agglutination-titre (reaction), with an artificial typhoid immune serum, is known, is carefully mixed, according to the method described on p. 116. If agglutination does not take place at once, the inoculated tubes are kept one hour at 37° C. and again examined. They are examined macroscopically for clumping, in the manner described on p. 116.

It is always necessary to make, simultaneously, controls with the salt solution used as diluent and with normal human serum. The solution in the control-tubes must remain evenly turbid during the period of observation. It is always necessary to titrate the serum—that is, to determine in how great a dilution it still causes agglutination. In regard to the value of the results of this test, it must be remembered that the serum of healthy persons who have never had typhoid fever may cause the agglutination of typhoid bacilli. Experience has, however, shown that this is true only when the serum is highly concentrated. When the test is carried out in the above-described manner, normal serum does not cause agglutination in dilutions over 1 : 50. Therefore, if agglutination can be seen macroscopically in the inoculated tubes containing dilutions over 50 within, at the longest, one hour's stay in an incubator at 37° C., while the controls (with sodium chloride and normal serum in a dilution of 1:50) appear homogeneous, it can be assumed that in all probability the patient has typhoid fever, or has recently had it.

In reporting the result of the agglutination test it is not sufficient to speak of a positive or negative reaction, but rather the limit of the agglutinating power of the serum, its titre (potency), and the method by which the latter is estimated, should be given. The latter is necessary, because a number of investigators establish the appearance of agglutination microscopically with the high power, and not macroscopically, and consider the clumping of a few bacilli as evidence of agglutination. These authors must naturally, in order to avoid mistakes, set the lowest limit of the agglutination much higher (1:100) than is necessary with the macroscopical examination.

The diagnostic value of the *Widal* reaction is still further handicapped by the fact that it rarely appears at the beginning of the disease, and in a number of cases is absent during its entire course. As a rule the agglutinins cannot be detected until during the second week of the disease. From this it follows that a negative reaction never excludes the possibility of typhoid fever. The agglutination test cannot replace the direct detection of the bacilli. The appearance of agglutinins in the blood is to be considered merely as a symptom of typhoid fever. Their presence strengthens the diagnosis, but their absence does not shake it.

Ficker has recently endeavored to simplify the execution of the agglutination test in order to place it in the hands of the practitioner who has no laboratory equipment at his disposal. His "typhoid-diagnosticum," which replaces the living typhoid culture, consists of a mixture of dead typhoid bacilli. It is a slightly turbid, sterile fluid, which keeps a long time fit for use if kept cool in the dark, and if shaken from time to time. It must always be shaken before using.

"The test is carried out in the following manner: A dilution of 1:10 of the serum to be tested with sterile 0.85 per cent. sodium chloride solution, is made by means of a graduated pipette, and, for example, 0.2 and 0.1 cc of this dilution are placed in conical test-tubes. To tube 1, 0.85 cc of the 'diagnosticum' is added; to tube 2, 0.9 cc. A third tube receives 1 cc of the 'diagnosticum' without the addition of serum (control). The tubes are closed with a cork or rubber stopper, the contents thoroughly mixed, and set aside at room-temperature and protected from the light. The result is evident after ten, twelve, or fourteen hours. The determination of the result must not be postponed more than twenty hours. The observation of the contents of the tubes is simplified if they are examined against a black background, or if the outspread hand is held 5 to 10 centimetres behind the tube, which is raised to the level of the eye, and between it and the source of light (window). Positive agglutination is made evident by clarification, and simultaneous clumping of the agglutinins contained in the specimen, which is particularly well seen owing to the use of a conical test-tube."

The control, which contains the "diagnosticum" alone, must, of course, remain evenly turbid.

The agglutination test is not applicable for the early diagnosis of other infectious diseases, as cholera and plague.

The examination of the blood for bacteriolysins has been used in the so-called *Pfeiffer's* test (cf. p. 122) for the recognition of convalescing cases of typhoid and cholera. The detection of the specific bacteriolysins has not been used in the diagnosis of new cases up to the present time. Stern[1] has recently used it in the diagnosis of typhoid fever. His results, however, have not as yet been tested with a large amount of material. He tested the serum of patients suspected of having typhoid fever with the aid of the test-tube reactions of bactericidal substances, according to the methods worked out by *Ehrlich* and his scholars.

It is determined what the smallest dose is in which the serum to be examined still has bactericidal action. For this purpose a constant quantity of typhoid bacilli and of normal complementary serum is added to decreasing quantities of serum which has been rendered inactive.

Stern used 0.5 cc of a fresh 1: 10–15 dilution of normal rabbit serum as complement; for inoculation, 0.5 cc of a dilution of 1: 5,000 of a twenty-four-hour typhoid bouillon culture. The sera are diluted with 0.85 per cent. sodium chloride solution; the culture is diluted with bouillon.

The test is performed in the following manner: The serum to be examined is rendered inactive by heating for half an hour on a water-bath at 55° C. Quantities of 1.0, 0.3, 0.1, 0.03, 0.01 cc, etc., are placed in a series of tubes by means of a sterile 1 cc graduated pipette. If it is suspected that the serum is of high potency, dilutions of 1:50–100 are made at once. In addition, each tube receives 0.5 cc of a 1:12 dilution of fresh rabbit serum, and 0.5 cc of a 1: 5,000 typhoid bouillon culture. The tubes are then filled to the same level—2 cc—with 0.85 per cent. sodium chloride solution. It is necessary to make the following controls:

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[1] *Berliner Klin. Wochenschr.*, 1904, No. 3.

Two controls of the typhoid culture (I and II), each of which contains 1.5 NaCl + 0.5 cc of a 1:5,000 typhoid bouillon culture.

A plate is made at once from Control I, another after three hours at 37° C. from Control II.

Controls III and IV establish the inactivity of the maximal dose used of the immune serum which has been rendered inactive, and of the complementary serum alone.

Control III: 1.0 of immune serum which has been rendered inactive. +0.5 cc of 1:5,000 typhoid culture +0.5 cc NaCl.

Control IV: 0.5 rabbit serum +0.5 cc of 1:5,000 typhoid culture +1.0 cc NaCl.

Controls V and VI test the sterility of the maximal quantity of the two sera used.

Control V: 1.0 cc of the inactive immune +1.0 cc NaCl.

Control VI: 0.5 cc of rabbit serum +1.5 cc NaCl.

All the tubes, with the exception of Control I, are, after being shaken, placed for three hours in an incubator at 37° C. After this length of time they are again shaken, and agar plates made from them by mixing their contents with melted agar which has been cooled to 42° C., and pouring the latter into Petri dishes. The plates are placed, inverted, in an incubator, and remain there until the following day, when it is determined how many colonies have developed in the individual plates. The estimation is made according to the following scheme: 0, or almost 0, about one hundred, a few hundreds, thousands, many thousands, innumerable colonies. In the examination of the plates it is noticed that those containing the largest quantities of immune serum show the most colonies. This fact is due to the diversion of the complements by superfluous immune bodies.

A distinct bactericidal action is present only when the controls tally, and there is reduction of the colonies from innumerable, or many thousands, to 0, or very few.

Still further, the test is to be considered as positive only when the lowest limit of the active serum dilution has been reached—that is, when the last plates show an increasing number of colonies (*Neisser*).

The Serum Diagnosis of Syphilis According to Wassermann

The positive result of the *Wassermann* reaction indicates that the individual furnishing the examined serum is or has been infected with syphilis. The theory of the reaction is based on the observation that a combination of antigen and antibody will bind complement, whereas neither antigen nor antibody alone possesses any or a very slight affinity for complement.

A hemolytic system (red blood-cells + serum, obtained from a rabbit previously injected with red blood-corpuscles serves as an indicator for the complement fixation. If we add a hemolytic system to a solution containing antigen + antibody and complement, hemolysis does not take place, because of the complement entering into the antigen + antibody complex, whereas hemolytic amboceptor and complement are required for the production of hemolysis. If, on the other hand, the solution contains either antigen or antibody and complement, the complement cannot be fixed, and it remains free to produce solution of the red blood-corpuscles of the added hemolytic system. The absence of hemolysis, therefore, shows a positive reaction, and its presence indicates a negative reaction.

In the examination for antibodies, which are traceable to luetic infection, blood serum is used; in parasyphilitic diseases of the central nervous system lumbar fluid is used. A specially prepared extract of the livers of congenitally syphilitic infants is used as antigen, the livers having been previously tested as to their fitness for this purpose. The method of procedure is as follows:

The liver extract is added to the previously inactivated serum, complement consisting of guinea-pig serum is then added, and the mixture is put into the incubator for one hour, to permit the action between antibody, antigen, and complement to take place. On the expiration of this time, the hemolytic system is added, and the tubes are again allowed some time in the thermostat, and thereupon are set in the refrigerator overnight. The result of the test may then be determined. If the examined serum contains antibodies, that is, if the result be POSITIVE, hemolysis *has not* taken place, the red blood-corpuscles are collected at the bottom of the tube and the supernatant liquid is colorless; in the absence of antibodies, that is, if the result is NEGATIVE, this is shown by the fact that solution *has* taken place, the tube contains no sediment of red blood-corpuscles, but the entire almost clear liquid has become red, colored by the dissolved blood pigment.

Method of Applying the Reaction.—The blood necessary for the reaction is obtained by a venous puncture, the median basilic vein being the best for the purpose, or by the use of a cupping cup. Six to eight cc are taken from adults, and 1½ to 2 cc from children. The blood is received into sterile centrifuge tubes, and after clotting, it is separated from the walls of the tube by means of a sterile needle and centrifugalized to obtain the required serum. The separated serum is decanted and immediately inactivated (complement destroyed by heating it on a water bath to 56° C. for half an hour).

Two control sera, similarly inactivated, are always examined simultaneously with the suspected serum, one from a known syphilitic serum, rich in antibodies, and the other a known normal serum. The serum must be examined in as fresh a state as possible, as syphilitic serum which has been kept for a time may lose its property of binding complement, and vice versa; normal serum may develop this property. Two specimens of the suspected serum, namely, 0.2 cc and 0.1 cc, and one each of 0.2 cc of the control sera, are employed.

Antigen.—If the liver furnishing the antigen is not to be used at once, it may be kept in a *Morgenroth* refrigerator in a frozen condition.

Preparation of the Aqueous Extract (*Wassermann* and *G. Meyer*). The liver is cut into small particles, with a knife and scissors, mixed with four parts of physiologic salt solution, to which phenol in the proportion of 0.5 per cent. is added, and the mixture agitated in the shaking apparatus for twenty-four hours. For example, 360 cc salt solution (0.85 per cent.), 100 gms. liver, 40 cc phenol, 0.5 per cent. The resulting mass is centrifugalized until a perfectly clear extract is obtained.

Alcoholic Extract.—*Michaelis* furnishes the following formula for its preparation: "The liver is minced in a mortar, ten volumes of absolute alcohol are immediately added, and the mixture shaken with the aid of glass beads for ten to twelve hours. At the expiration of twenty-four hours, the clear liquid is removed from the sediment with a pipette, and preserved in the refrigerator, to be used as stock solution (antigen). For each test of the reaction, a freshly prepared dilution of the stock solution with four parts of physiologic salt solution is employed. This alcoholic stock solution forms a slightly milky emulsion with the aqueous solution. Its slight alcoholic content does not interfere with the reaction. On allowing this milky emulsion to stand, a flocculent precipitate gradually settles on the bottom; the clear supernatant liquid is then ineffective, as the active principle is fixed in the precipitate and the emulsion must be well shaken to render it active again. One cc of this diluted emulsion is, as a rule, employed for the reaction, although considerable latitude as to the quantity of liver extract is permitted.''

Alcoholic extracts of normal organs, prepared in the above-described manner, have been recommended as practicable antigen. *Fleischmann* found four out of five extracts of normal livers available. *Michaelis* has used a very effective alcoholic extract of normal (human) hearts. It is better, however, at the present stage, at least, to use the extract of syphilitic livers in order to obtain unobjectionable results.

Previous to its employment, each extract must be tested as to its availability by comparison with a known syphilitic and a known normal serum, as available extracts are not always obtained, even from syphilitic fetal livers.

Antigen is considered particularly efficacious if 0.1 cc + 0.1 cc of syphilitic serum inhibits hemolysis completely. Antigens, of which 0.2 to 0.4 cc + 0.1 to 0.2 cc of luetic serum are required for complete inhibition, may also be used, provided they do not produce inhibition either with normal sera or by themselves in double amount (*G. Meyer*).

For the preliminary test of the extract, following the suggestion of *Meyer*, the smallest amount of extract, which by itself will bind complement, is determined by testing with 0.2, 0.4, 0.6, 0.8 and 1.0 cc of antigen; and furthermore, the smallest dose which will cause complete inhibition with 0.1 cc of luetic serum is noted.

The extracts, when kept cool and dark, often remain serviceable for several months, but they may suddenly become ineffective; for this reason it is imperative that their efficacy be controlled at each test with a known luetic and a known normal serum. In making the test, two correspondingly different doses of the extract and the serum are used, as, for instance, 0.2 cc of antigen + 0.2 cc of serum, and 0.1 cc of antigen +0.1 cc of serum; the amounts used depend upon the result of the preliminary examination.

Complement.—Fresh guinea-pig serum serves as complement. One cc of a 10-per-cent. dilution in physiologic salt solution is required. The serum may be conserved for a short time (not more than forty-eight hours) in the ice-chamber. Guinea-pig blood may be obtained by heart puncture.

Hemolytic System.—Red blood-corpuscles (erythrocytes) of the sheep, and serum obtained from a rabbit previously treated by repeated injections of washed sheep's blood-corpuscles, constitute the hemolytic system. The sheep's blood is obtained by puncture of the jugular vein and received in salt solution. The blood-corpuscles are separated by centrifugalizing, and washed several times with physiologic salt solution. The blood-corpuscles are shaken with the salt solution in the centrifuge tube, then centrifugalized and the supernatant liquid is poured off; this is repeated several times. For the test, 1 cc of a 5-per-cent. suspension of blood-corpuscles in physiologic salt solution is used.

The hemolytic serum keeps well for some time, if put into a dark bottle and placed in the refrigerator. For the test, 1 cc of a dilution, which corresponds in solving power to a serum capable of dissolving a two and a half to threefold dose of the serum, is employed.

Its titre must be determined before instituting the reaction. For trial-tests, *G. Meyer* recommends one and one-half, two- and threefold strengths of that dilution with which good results were obtained at the last test. In order to adapt the result of the trial-test to the conditions of the actual test, the complement, with the addition of the blood-corpuscle serum mixture and 2 cc of physiologic salt solution is placed in the incubator for one hour.

If the titre of the serum has remained unchanged, complete hemolysis will be found to have taken place in the tube containing the one and one-half dilution within half an hour, and in the tube containing the twofold dilution at the latest within one hour. The threefold dilution is, as a rule, completely hemolyzed within two hours, but that result need not be awaited in order to proceed with the reaction.

For the Determination of the Reaction the Following Are Necessary:

1. Antigen in two doses, i.e., 0.1 and 0.2.
2. The serum to be examined, 0.5 cc.
3. Known luetic serum, 0.4 cc.
4. Known normal serum, 0.4 cc.
5. Fresh guinea-pig serum (1:10 NaCl solution).
6. Five per cent. suspension of sheep's erythrocytes in physiologic salt solution.
7. Rabbit serum, hemolytic for sheep's erythrocytes.
8. Physiologic salt solution.

The test-tubes should have a capacity of at least 5 cc, and are eventually filled up to that amount with physiologic salt solution. For the purpose of avoiding with certainty any possible source of error, a row of control test-tubes are employed in addition to the tubes containing the suspected sera (tubes I and II).

1. A control with a double dose of antigen, to prove that the antigen itself produces no fixation (tube III).
2. The positive control: Antigen and known luetic serum (tube IV).
3. The negative control: Antigen and known normal serum (tube V).

The controls in tubes III, IV, and V serve to demonstrate the fitness of the antigen.

4. Controls to show that the sera themselves do not fix complement (tubes VI, VII, and VIII).
5. A tube containing NaCl solution, complement and blood-corpuscles is employed to prove that the complement itself possesses no hemolytic properties (tube IX).
6. Control of the hemolytic system (tube X).
7. A tube containing salt solution and red blood-corpuscles in NaCl solution (tube XI).

In making the test, the tubes containing serum, antigen, and complement are placed in the incubator for one hour to produce fixation of the complement. Thereupon the sheep erythrocytes and the hemolytic serum, both of which have previously been kept for about one-quarter to one-half an hour at a temperature of 37° C., are added to each tube.

The course of the reaction must now be watched carefully; as soon as hemolysis is complete in the control-tubes, the test-tubes are taken from the thermostat and placed on ice for twenty hours.

The determination of the reaction with an antigen, the efficient dose of which has been determined as 0.1

cc, and giving a positive result, is illustrated by the following table:

Tube No.	Antigen.	Serum.			Complement.	Salt Solution.	5% Suspension of Blood-Corpuscles.	Hemolytic Serum.	Result.
		To Be Examined.	Known Positive.	Known Normal.					
I...	0.2	0.2	...	...	1.0	1.6	1.0	1.0	Fixation.
II...	0.1	0.1	...	...	1.0	1.8	1.0	1.0	"
III...	0.4	...	...	...	1.0	1.6	1.0	1.0	Hemolysis.
IV...	0.2	...	0.2	...	1.0	1.6	1.0	1.0	Fixation.
V...	0.2	...	...	0.2	1.0	1.6	1.0	1.0	Hemolysis.
VI...	...	0.2	...	...	1.0	1.8	1.0	1.0	"
VII...	...	...	0.2	...	1.0	1.8	1.0	1.0	"
VIII...	...	...	...	0.2	1.0	1.8	1.0	1.0	"
IX...	...	...	...	...	1.0	3.0	1.0	...	No hemolysis.
X...	...	...	...	...	1.0	2.0	1.0	1.0	Hemolysis.
XI...	...	...	...	...	...	4.0	1.0	...	No hemolysis.

CHAPTER X

EXAMINATION OF FLUIDS OBTAINED BY PUNCTURE

A.—General Characteristics and Chemical Examination

1. ***Transudates.***—Transudates are light yellow with a tinge of green usually transparent, acid in reaction, and deposit fibrin on standing, as a rule, in the form of a moderately gelatinous or membranous clot. The precipitation of the clot may be somewhat hastened by the addition of a small amount of blood. This slight admixture of blood usually takes place at the time of puncture.

The specific gravity of transudates is comparatively low, and varies with the location of the transudate. According to the investigations of *Reuss* the specific gravity of transudates varies from 1,005 to 1,015. The highest specific gravities (to 1,015) are found in hydrothorax, the lowest in hydrocephalus.

The albumin contained in transudates is slight in comparison with that contained in exudates, and rarely exceeds 2.5 per cent.

2. ***Exudates.***—Exudates show greater variations. A distinction is made between serous, hemorrhagic, purulent, and sanious exudates. The color, transparency, and consistency of these products of inflammation also vary correspondingly. The specific gravity is almost always above 1,018. The albumin contained rarely sinks below

2.5 per cent. However, the differences in the specific gravity and in the albumin are not so constant as to render it possible to differentiate between exudates and transudates in every case by these two factors alone. Transudates are occasionally found whose albumin exceeds the lower limit of the albumin contained in exudates, and *vice versa*. It has recently been claimed that exudates differ from transudates in that they contain an albuminoid body (according to *Umber*, seroso-mucin) which is precipitated by acetic acid. The presence of this albuminoid substance is detected by the fact that a solution clarified by filtration shows a marked turbidity, or throws down a precipitate, when rendered distinctly acid with acetic acid.

3. ***Ovarian Cysts.***—The contents of ovarian cysts are usually viscid and mucoid in consistency, light yellow, and occasionally dirty brown or yellowish-green in color. The specific gravity varies greatly (between 1,005 and 1,050). The presence of peculiar albuminoid substances, of which pseudo-mucin (also called para-albumin or metalbumin) is most frequently found, is characteristic of the contents of ovarian cysts. Pseudo-mucin is not precipitated by acetic acid, nitric acid, or boiling, but is precipitated by alcohol, and thus differs widely from mucin and albumin. The ordinary varieties of albumin (albumin, globulin) are present in varying quantity in the contents of ovarian cysts.

Pseudo-mucin is detected in the following manner: (1) 25 cc of the fluid are treated with a few drops of an alcoholic solution of rosolic acid, heated to the boiling-point, and treated with dilute (decinormal) sulphuric acid, until the change of color to yellow indicates that the fluid is faintly acid. It is again heated to the boiling-point and filtered. If the filtrate is clear, no pseudo-mucin is present. If the filtrate is turbid, it suggests pseudo-mucin, but does not determine it with certainty, since the turbidity may be due to albumin which has not been entirely removed. The following test must be carried out to confirm a positive reaction: (2) 10 to 15 cc of the fluid (depending upon its specific gravity) are freed from albumin by boiling, and precipitated with three times the volume of 95 per cent. alcohol. The flaky precipitate is collected on a filter, dried between filter-paper, and dissolved in water. In the presence of pseudo-mucin, an opalescent solution is produced. Acetic acid is added, and the solution filtered. To the filtrate one-quarter of its volume of 25 per cent. hydrochloric acid is added (4 parts filtrate, 1 part 25 per cent. hydrochloric acid). The solution is then heated in a water-bath, five to ten minutes (until it becomes brownish-yellow or brown). After it has cooled it is neutralized with concentrated sodium hydrate, and tested with *Fehling's* and *Nylander's* tests. If pseudo-mucin is present, both tests give a positive result.

4. **Hydronephrosis.**—The contents of hydronephroses usually resemble dilute urine, but their appearance may be altered by the admixture of pathological constituents (mucus and pus). The detection of both urea and uric acid suffices for the identification of a fluid as hydronephritic. It must, however, be remembered that these urinary constituents may be absent in old, thoroughly closed cysts. Concerning the detection of urea and uric acid, cf. p. 125.

5. **Echinococcus Cysts.**—Echinococcus fluid is usually clear, of low specific gravity, alkaline or neutral in reaction, and contains considerable sodium chloride and no albumin, or only a very slight quantity. Succinic acid and its salts are considered as characteristic constituents

of echinococcus cysts, since they have frequently been found in them in small quantities. Succinic acid is detected in the following simple manner:

The fluid is evaporated down to a syrupy consistency, acidified with hydrochloric acid, and extracted with ether containing alcohol. The ether is removed by evaporation in a water-bath, and the succinic acid remains as a crystalline residue. Microscopical examination reveals hexagonal plates or monoclinic prisms. When heated in a platinum dish, choking fumes, which have a peculiar odor, are given off. An echinococcus cyst can be diagnosed with absolute certainty, however, only by microscopical examination (detection of hooklets or membrane).

6. ***Pancreatic Cysts.***—The contents of pancreatic cysts are usually hemorrhagic. They contain, as a rule, albumin (serum albumin), and occasionally mucin. The presence of a diastatic ferment can usually be detected, but is of little value in diagnosis, since diastase may appear in other fluids of the body. The detection of trypsin is much more important, and is accomplished by treating the fluid with milk, placing it for some time in an incubator, precipitating the casein, and testing the filtrate for the biuret reaction. A positive result of the test indicates that the fluid can digest albumin in the presence of an alkaline reaction.

This proves that the fluid is from a pancreatic cyst, since, as yet, a ferment capable of peptonizing in the presence of an alkaline reaction has been detected in no other aspirated fluid. This ferment may, however, be absent in old encapsulated cysts.

7. ***Cerebro-Spinal Fluid.***—In healthy persons the fluid obtained by lumbar puncture is colorless, clear, and of low specific gravity (1,003 to 1,006). Its chemical com-

position is in no way characteristic, so that only its microscopical and bacteriological examination are of diagnostic value.

B. Microscopical Examination

The fluid is allowed to stand for some hours in a conical glass and a few drops of the precipitate removed with a pipette and examined microscopically. If the quantity of fluid is small and it contains but little suspended matter, it is centrifugalized. Unstained smears are first examined. Stained smears are best prepared by spreading the sediment, with a small pipette, in a thin layer on a cover-glass, allowing it to dry in the air, and simultaneously fixing and staining according to *May* and *Gruenwald* (cf. p. 339).

Transudates contain but few solid constituents, a few leucocytes in a state of fatty degeneration, and isolated, flat epithelial cells. Serous exudates contain, as a rule, in addition to the fibrin clot and red blood-corpuscles (the latter usually become mixed with the fluid at the time of puncture), many leucocytes, and epithelial cells in a state of granular or fatty degeneration, which frequently show large vacuoles. In the presence of neoplasms (cancer) the number of cells with vacuoles is markedly increased; they are in an advanced stage of fatty degeneration, and lie in large groups. Such groups of cells must awaken suspicion of neoplasms if they are found in a hemorrhagic exudate. French authors accredit diagnostic value to the different varieties of leucocytes contained in the fluids. They have originated the following so-called cytological scheme:

1. Excess of lymphocytes—i.e., mononuclear leucocytes—indicates that the exudate is tubercular.
2. Excess of polynuclear and eosinophilic leucocytes

indicates that the exudate is infectious but not tubercular.

3. Excess of endothelial cells indicate that the fluid is of mechanical origin (transudate in cardiac, renal, and hepatic diseases).

This scheme holds in a large number of cases, but, unfortunately, not in all.

In the examination of echinococcus fluid the detection of hooklets and membrane is of much greater diagnostic value than the chemical detection of succinic acid. In ovarian cysts, in addition to the red and white blood-corpuscles, cells in a state of fatty degeneration, and having vacuoles, are found. Cylindrical and ciliated epithelial cells, goblet cells, and colloid concretions, are characteristic of ovarian cysts.

Cerebro-spinal fluid is normally clear, and contains only occasional solid constituents (leucocytes). In disease the number of solid constituents is very frequently increased.

C. Bacteriological Examination

1. Collection of Material for Examination

Material for examination is obtained by means of exploratory puncture, or occasionally during the therapeutic measures (operation for empyema, lumbar puncture, etc.).

For collecting intraperitoneal fluids a trocar is used, which is introduced, with the patient in the sitting posture, in the left side of the abdomen, half-way between the symphysis and the anterior superior spine of the ilium. The fluid is collected in a sterile flask.

Pleuritic effusions are collected for examination by means of exploratory puncture with a sterile syringe, of a capacity of 2 to 10 cc, and having a long (about 7 centimetres) steel needle, of medium weight. Immediately before aspiration the patient must be examined in the position in which the puncture is to be made, in order to determine the position of the exudate. The puncture is always made on the upper border of the rib, in widespread effusions on the left side, in the sixth or seventh; on the right side in the fourth or fifth intercostal space, between the anterior and midaxillary lines; at the back in the eighth or ninth intercostal space.

In meningeal effusions the material for examination is obtained by means of lumbar puncture, suggested by *Quincke.* There are two sets of instruments for this operation in use. One suggested by *Quincke*, the other by *Kroenig.* For spinal puncture the patient should be on the side, with the back curved and the thighs drawn up on the body. The needle is introduced between the fifth vertebra and the sacrum, a few millimetres from the median line. *Quincke* recommends the third or fourth interarticular space as the site of the puncture. The hiatus sacro-lumbalis is, however, better adapted for diagnostic purposes, since, ''owing to the conical form of the lower portion of the arachnoid sac, it allows a natural sedimentation of the histological and bacteriological substances'' (*Kroenig*). The evacuation, which must always be controlled with a manometer, must be stopped as soon as the pressure sinks below 50 millimetres. The fluid is collected in sterile test-tubes, in quantities of 10 to 20 cc per tube.

2. Method of Examination

1. ***Microscopical Examination.***—If the fluid has a purulent character, a smear is either made from it at once, or a portion is centrifugalized, and the sediment used for the preparation of stained smears. The smears are stained

with dilute carbol-fuchsin or methylene blue, according to *Gram*, and for tubercle bacilli. For the detection of the latter the sedimentation method described under Examination of the Sputum may be used. If the fluid contains blood, potassium hydrate must be added before it has coagulated. If the fluid is serous, it is advisable to collect as much as possible of it, since the number of micro-organisms contained is frequently very small, and the possibility of their detection increases with the quantity of material obtained for examination. Serous fluids are allowed to stand six to twenty-four hours in the receptacle in which they are collected, in an ice-chest. During this time a clot resembling a spider's web frequently forms, which collects the bacteria present in the fluid. This clot is removed *in toto* with a platinum wire, carefully spread on a slide, dried in the air, fixed, and stained. If such a clot is not formed, as large a portion of the fluid as possible is centrifugalized in the same centrifuge-tube. Stained smears are made from the sediment. It is not advisable to fix these smears in the flame, but rather for three minutes in alcohol and ether āā. The method of *May* and *Gruenwald* yields good results (cf. p. 339).

Jousset has recently recommended his method of inoscopie (*ἴς ἰνός* = fibrin) in the examination of serous fluids, especially for tubercle bacilli, but also for other bacteria. After the clot has formed it is separated from the fluid by filtration, washed with distilled water, and treated with 10 to 30 cc of the following mixture:

Pepsinæ		2.0
Glycerini pur., Acidi hydrochlorici, 22° Baumé .	āā	10.0
Natrii fluorat		3.0
Aqua dest.	ad	1000

This digestive fluid, containing the clot, is placed for two to three hours in an incubator at 37° C. During this time the fibrin and the cell protoplasm are dissolved, while the bacilli remain intact, and lose none of their staining characteristics. The fluid is then centrifugalized, and specimens made from the sediment. If no clot forms spontaneously, *Jousset* suggests artificial coagulation by means of a proper medium (for example, plasma of horse-blood).

Frequently the pathogenic bacteria are present in the fluids in such small numbers that a number of specimens must be examined before any bacteria are found. In the examination of cerebro-spinal fluid for tubercle bacilli, *Slawyk* recommends that the last fluid which escapes at the time of puncture be examined, since it contains a larger number of tubercle bacilli. If no micro-organisms are detected microscopically, cultural procedures and, if necessary, animal inoculation must be resorted to for their detection. In a number of the cases, especially in old effusions, these methods also fail.

2. ***Cultural Procedures.***—The choice of the culture media depends upon the variety of micro-organisms found in the smears. When the microscopical examination is negative, various culture media must be used. Ordinarily, agar, glycerine-agar, serum media, and blood-agar (for the cultivation of influenza bacilli) are used. The culture medium, contained in Petri dishes, is inoculated in the usual manner with the material to be examined. If, as is frequently the case with serous fluids, no bacteria, or very few, are detected microscopically, the sediment obtained by centrifugalization is used for inoculation. In the cultural examination of serous fluids for tubercle bacilli, 30 to 50 drops are allowed to run into blood-serum or glycerine-agar tubes. The excess of the fluid is allowed

to evaporate in the incubator at 37° C., and the application of rubber caps to prevent drying of the culture media is postponed until but little fluid remains.

3. ***Animal Inoculation.***—Animal inoculation serves the purpose first of detecting micro-organisms in the fluids, and second of identifying the bacteria found in the smears, or by cultural methods. The choice of the test-animals and the method of inoculation must be suited to the pathogenic bacteria whose presence is suspected, or whose identification is desired. For example, white mice are used for the identification or detection of streptococci or pneumococci, and guinea-pigs for tubercle bacilli (cf. Examination of the Sputum).

In examining for tubercle bacilli, animal inoculation will succeed more frequently than microscopical or cultural methods. In the examination of serous fluids, either the above-mentioned coagulum is used for inoculation, or a considerable quantity of the exudate—at least 4 cc—is injected into the animal. In purulent exudates the sediment obtained by centrifugalization is used.

3. The Most Important Bacteriological Findings

1. ***Peritoneal Exudates.***—The bacteriological findings in acute peritonitis depend principally upon the locality from which the inflammation extends. Mixed infections are very frequent. In peritonitis of intestinal origin, principally bacilli belonging to the group of *Bacterium coli* are found, together, as a rule, with other micro-organisms of the intestinal flora, as staphylococci, *Proteus vulgaris*, *Bacillus pyocyaneus*, etc. Peritonitis extending from the female genital organs is most frequently caused by gonococci; puerperal peritonitis by streptococci and other pyogenic bacteria. In peritonitis in which the infection has reached the peritoneum by means of the circulation, strep-

tococci and pneumococci have been most frequently found; in peritonitis following operations, streptococci. Typhoid bacilli, actinomyces, pseudo-diphtheria bacilli, and bacilli resembling tetanus bacilli have also been found in peritoneal exudates. Finally, the tubercle bacillus is of great importance as the exciting cause of chronic peritonitis.

2. **Pleuritic Exudates.**—Pleuritic exudates are also of varying origin. Tubercle bacilli, as well as all the pyogenic micro-organisms, may be the exciting cause of pleuritic effusion. Serous exudates are by far most frequently tubercular. A negative microscopical and cultural examination should arouse strong suspicion that they are tubercular. The inoculation of guinea-pigs must always be used in the diagnosis of such cases.

In purulent exudates streptococci are most frequently found; next, pneumococci, staphylococci, and tubercle bacilli; more rarely, influenza bacilli and *Micrococcus tetragenus*. Typhoid bacilli have been detected in pleuritic effusions in the course of typhoid fever. Pleuritic effusions accompanying pneumonia frequently contain pneumococci, either alone or with staphylococci.

Sanious exudates contain, in addition to the pyogenic cocci, bacteria of decomposition and anaerobic varieties.

3. **Meningitic Effusions.**—Normal cerebro-spinal fluid is clear, free from bacteria, and contains only occasional lymphocytes and epithelial cells. The pathological effusion is colorless and clear in the cerebral œdema of chlorosis, uræmia, cerebral tumors, and in serous meningitis (*Kroenig*).

In tubercular meningitis the fluid is also usually clear, but occasionally somewhat opalescent, and frequently contains many leucocytes. In the early stages of the disease the polynuclear leucocytes seem to prevail, and in the later stages the mononuclear. In acute non-tubercular meningitis the character of the fluid varies according to the intensity of the process, even when the cause is the same; it may be serous, fibrinous, fibrino-purulent, or purulent.

The *Diplococcus intracellularis meningitidis* "*Weichselbaum*" and the *Diplococcus pneumoniæ* have been detected as the exciting cause of acute, primary, epidemic, and sporadic cerebro-spinal meningitis.

Concerning the *Diplococcus pneumoniæ*, cf. p. 48.

The *Diplococcus intracellularis meningitidis* appears usually as a diplococcus or tetracoccus; the cocci are somewhat flattened on the inner side, and have therefore a hemispherical or coffee-bean form. They often vary considerably in their size and staining qualities, so that in the same smear smaller and larger feebly stained cocci (degeneration forms) are found beside the normal cocci. They resemble gonococci in form and arrangement, but are larger. Like gonococci, they frequently lie in the exudate in groups within the pus corpuscles. Their staining characteristics also agree with those of gonococci; they stain easily with dilute aniline dyes, and are decolorized by *Gram*.

4. ***Cultural Behavior.*** — The *Diplococcus* "*Weichselbaum*" grows best at a temperature of 36° to 37° C. Upon agar, gray to grayish-white colonies are formed within twenty-four hours, which are 1 to 2 millimetres in diameter, have smooth or wavy margins, and are, when examined against the light, transparent and yellowish.

Upon serum plates the growth is more luxuriant; the grayish-yellow colonies are moistly glistening and viscid, like those of the diplococcus of *Friedlaender*.

Their cultivation from meningitic exudates succeeds best on serum, but it occasionally fails even on this medium.

Animal inoculation is not used for diagnostic purposes. White mice die in twenty-four to forty-eight hours following the intrapleural injection of a comparatively large quantity of a culture. Post mortem, the diplococci are found both within and without the cells in the pleuritic or peritoneal exudate.

Pneumo-, staphylo-, and streptococci, influenza bacilli, the diplobacilli of *Friedlaender*, *Bacterium coli*, and plague bacilli have been reported as the exciting cause of cerebrospinal meningitis secondary to infectious disease.

CHAPTER XI

BACTERIOLOGICAL EXAMINATION OF DISEASES OF THE SKIN

Purulent Affections of the Skin

Material for examination is, as a rule, obtained by puncture with a sterile needle, or by incision. If the bacteriological examination happens to be undertaken at the time of an operation, especial care must be taken that the material for examination does not come in contact with disinfectants.

The material is usually examined microscopically and by means of cultures. Animal inoculation is used in case these two methods fail, or to identify bacteria which have been cultivated.

As the exciting cause of furuncular processes, *Staphylococcus aureus* or *albus* can almost always be detected microscopically, and by cultivation upon the usual culture media. In the pus of panaris (whitlow), in addition to staphylococci, streptococci, and more rarely *Bacterium coli*, may be found. In acute abscesses and phlegmona, in addition to the above-mentioned pyogenic bacteria, pneumococci, typhoid bacilli, etc., may be found. In large abscesses the detection of micro-organisms frequently fails in pus taken from their centre, while in the periphery, in the so-called abscess membrane, their detection is easy. In the so-called cold abscesses the bacteria can, as a rule, be detected neither microscopically nor by cultural means. The presence of tubercle bacilli in them can also, as a rule, be detected only by inoculating guinea-pigs with the pus.

In the pus of gas phlegmona, bacilli belonging to the group of *Bacterium coli* and *Bacterium lactis aerogenes*, as well as anaerobic bacteria (*B. emphysematosus*), may be found, in addition to the ordinary pyogenic bacteria.

In multiple abscesses developing in the skin and muscles, in the course of glanders, it is, as a rule, impossible to detect the *Bacillus mallei* microscopically. In suspected cases cultures are planted upon glycerine-agar and potato, and animal inoculation is used. The potato cultures are very characteristic. After two days, a honey-yellow coating can be seen, which, after a week, is brownish-red, and surrounded by a slightly greenish, shimmering zone. Upon glycerine-agar transparent grayish colonies are seen.

Bacilli mallei are small, slim, slightly curved, non-motile rods, about the size of tubercle bacilli. They do not stain with dilute dyes, but best with *Loeffler's* alkaline methylene blue.

Male guinea-pigs are used as test animals. The suspected material is injected into the peritoneal cavity in the median line above the bladder. After two to three days the testicles become swollen, which is a characteristic symptom of successful transmission of glanders. Potato cultures are inoculated from the diseased testicles.

Anthrax carbuncle, the so-called malignant pustule, is due to infection with anthrax bacilli. Lymph obtained from the deep portion of the suspected pustule is used as material for examination. The serous contents of the pustule are free from bacilli, since the latter lie about the papillæ, in the external portion of the corium. Specimens are stained with dilute methylene blue, according to *Gram*, and by one of the methods which serve to demonstrate capsules. Microscopical examination, however, yields positive results only for a short time following the formation of the carbuncle; later, the bacteria can be detected only by means of cultures or animal inoculation, which may also fail. Gelatine and agar plates are inoculated with the lymph. After twenty-four hours' growth, characteristic colonies of anthrax bacilli are seen. Staphylococci also frequently develop along with them. Further, white mice or guinea-pigs are inoculated by implanting the material to be examined in a pocket under the skin above the base of the tail. Bacilli which develop in the cultures are also identified by animal inoculation.

Anthrax bacilli (Plate XV, Fig. Z) are clear, cylindrical, non-motile rods, with rounded ends, and of varying length; they appear much larger in cultures than in the animal organism. They stain easily with dilute aniline dyes, and according to *Gram*. In stained smears the bacilli usually have a slight bulbous enlargement at their ends, and, at the same time, a slight concavity, so that when two bacilli lie end to end, a small hole is formed between the points of contact (bamboo-form). In the *Gram* specimen they are often unevenly stained, and appear granular. In specimens obtained from animal organisms anthrax bacilli possess a mucoid covering, the so-called capsule, which may be demonstrated by special staining methods (cf. p. 335).

Anthrax bacilli form centrally placed spores in the presence of free O, and at temperatures above 18° C.

Anthrax bacilli grow upon all the usual culture media. Upon agar and gelatine they develop very characteristic colonies. On examination with the low power, numerous spiral branches are seen extending from a centre composed of a non-transparent whorl of threads, which give the colony the appearance of a tangle of hair. Bouillon is not clouded *in toto*, but a sediment is formed. In gelatine stab cultures the bacilli grow along the stab canal and form delicate branches from it. Gelatine is liquefied; milk is coagulated.

White mice and guinea-pigs are used as test animals for diagnostic purposes. The animals die of anthrax septicæmia one to three days following subcutaneous inoculation. Post mortem, the spleen is found greatly enlarged. Bacilli are found only in small number in the heart's blood, but in great number in the capillaries of all the viscera, especially in the spleen and liver, and show, in microscopical specimens, the characteristic capsules.

Bacilli of malignant œdema must be considered in making a differential diagnosis. These are motile, have no capsule, and are absolutely anaerobic. Anthrax bacilli are distinguished from the saprophytes which form similar colonies (potato, and hay bacilli) by their typical morphological characteristics, and especially by the fact that they are pathogenic.

The Detection of Tetanus Bacilli (Plate XVI, Fig. a) ***in the Secretion of Infected Wounds.***—Tetanus bacilli are present in such small number in the secretion of the wounds that they cannot be detected microscopically. Cultural procedures also often yield a negative result. Animal inoculation is much more frequently successful. For this purpose, secretion from the wound, granulation tissue, or any foreign body found in the wound, is used, mice and guinea-pigs being inoculated in a pocket under the skin of the thigh. If the inoculated animals show no signs of tetanus within five days, the result should be considered negative. A positive result of animal inoculation is sufficient for diagnosis, it being unnecessary to grow the bacilli in pure culture.

Tetanus bacilli are slightly motile, slim rods, which in smears made from pure cultures, lie singly or arranged in threads of varying length. At room-temperature after eight to ten days, and at incubator-temperature after twenty-four to thirty hours, they form spores at one end, which give them the appearance of a drum-stick. Tetanus bacilli stain easily with dilute aniline dyes, and according to *Gram.*

Cultural Behavior.—Tetanus bacilli are anaerobic. They grow in the absence of air on all the usual culture media, especially well if grape-sugar (2 per cent.) is added. In symbiosis, with aerobic bacteria, they grow even in the presence of oxygen.

Cultivation of Pure Cultures According to Kitasato.—The material to be examined is planted upon agar tubes, which are placed for one to two days in the incubator, after which time tetanus bacilli having spores are present among the other bacteria. The mixed culture is now heated in a water-bath at 80° C. for about one hour, by which the other bacteria are killed, while the resistant tetanus spores remain capable of development. From these, anaerobic cultures are made in the usual manner (cf. p. 359).

After five days' growth on gelatine, small colonies with radiating branches have developed. Gelatine is liquefied. They develop much more rapidly upon agar. When examined with the low power, the delicate colonies appear as a maze of fine threads.

Animal Inoculation.—Mice and guinea-pigs are the most sensitive test animals, and are inoculated by means of a piece of wood, or the like, which is impregnated with the material to be examined and introduced, through a nick, under the skin. The first symptoms of tetanus appear in the muscles near the site of inoculation. The animals die with their hind legs stretched out. Tetanus bacilli can be detected microscopically and by cultural methods only at the site of inoculation.

The Detection of the Bacillus of Soft Chancre *(ulcus molle)*, discovered by *Ducrey*, is occasionally of practical value.

In smears made from the secretion of fresh ulcers and stained with *Loeffler's* methylene blue, borax-methylene blue, or polychrom-methylene blue, short, thick bacilli, which have rounded ends and frequently show polar staining, and which lie in groups, pairs, or singly, both within and without the cells, appear in addition to other microorganisms. They are decolorized by *Gram*. The picture which they present in sections, made from the periphery of the excised soft chancre, is characteristic. The bacilli frequently lie in long parallel chains, always outside of the cells, in the lymphatic spaces of the tissue, and everywhere a little beyond the border of the necrotic and within the living tissue. (Concerning the staining of sections, cf. p. 343.)

Bacilli of soft chancre do not grow upon the usual culture media. They may occasionally be cultivated upon blood-agar (2 parts liquefied agar, which has been cooled to 40° to 50° C., and 1 part rabbit blood and non-coagulated blood serum from the pus of the ulcer before it has ruptured through the skin covering it, and from inoculation chancres. After forty-eight hours' growth at 37° C., dark gray, glistening round colonies the size of a pin's head have developed, which may be shifted about upon, or lifted bodily from, the surface of the culture medium, with the platinum needle. The colonies are composed of polymorphous rods, which frequently lie in rows, and are seen to be non-motile when examined in a hanging-drop.

Transplantation on the human skin is used for the detection of *Ducrey's* bacillus. The side of the abdomen of the patient himself is used as the site of inoculation. The skin is scarified in several spots, and the secretion from the ulcer to be examined is rubbed in. After two to four days, secondary chancres develop, in whose secretion the bacilli are usually found in great numbers.

Tuberculosis of the Skin

Bacterioscopy has but little diagnostic value as regards tuberculosis of the skin, since, as in other chronic tubercular processes, the bacilli are usually present in such small numbers that the attempt to detect them microscopically often fails. They are most likely to be found in smears made from the secretion of tubercular ulcers, but their detection in such cases does not determine with certainty that they are the exciting cause of the disease, since tubercle bacilli may become located upon ulcerating surfaces without having an etiologic bearing upon the disease. Further, it must be remembered in making a differential diagnosis that other acid-fast bacilli are frequently found on the skin.

In *tuberculosis cutis verrucosa*, occasional tubercle bacilli are found in sections.

The bacilli, in skin affected by tuberculosis, are more likely to be detected by means of animal inoculation (subcutaneous inoculation of guinea-pigs) than by microscopical examination.

Diseases of the Skin Excited by Hyphomycetes (Dermatomycosis)

Collection of Material for Examination

For the collection of epidermal scales, the skin is either scraped with a dull, slightly moistened scalpel, or according to *Unna*, a piece of zinc oxide plaster or ordinary surgeon's plaster is laid upon the skin and pressed for a few minutes with the warm hand, then lifted, the scales which stick to it loosened with benzine, and freed with HCl alcohol from the zinc oxide which clings to them. Before further examination, the scales are placed in water, in which they become swollen.

Hairs are obtained for examination by epilation.

Small particles are scraped from the nails.

Microscopical Examination

The examination of unstained specimens very frequently suffices for diagnostic purposes. The material to be examined is placed upon a slide, and either rubbed with a 40 per cent. solution of potassium carbonate or a 10 to 15 per cent. solution of potassium hydrate, or crushed between two slides, and, after slight warming over the flame, is covered with a cover-glass and examined with the medium power (about 300). The oil immersion is used for the detection of the parasite of erythrasma.

Stained specimens are examined principally when the fungi are present in such small numbers that they escape detection in unstained specimens. Of the numerous staining methods which have been recommended, *Plauth's* modification of *Bizzozero's* method, and *Waelsch's* method (cf. p. 337) should be mentioned.

Hair must be freed from fat by several hours' immersion in a mixture of alcohol and ether before it is stained.

Cultural Procedures.—The most favorable culture media are grape-sugar, glycerine- and maltose-agar, *Sabouraud's* milieu d'épreuve (maltose, 4.0; peptone, 2.0; agar-agar, 1.5; aqua dest., 100.0), and wort-agar. In cultivating fungi from the horny layer of the skin, and from the hair and nails, *Král's* method may be used. As much material as possible is lightly rubbed, in a porcelain dish, with calcined infusorial earth; liquefied agar which has been cooled to 40° C. is inoculated with two to three loops of the infected earth and poured into plates. Dilutions may be made in the usual manner. After two to three days' growth the plates are examined with the low power, and the suspicious looking colonies removed and grown in pure culture. According to *Sabouraud*, the young cultures are transplanted upon the surface of congealed maltose-agar contained in 100 cc *Erlenmeyer* flasks. The layer of agar should be 1.5 centimetres thick. The flasks remain open in the incubator.

W. Scholz recommends the method used in the dermatological clinic in Breslau, which is especially suited to the cultivation of favus fungi from the hair.

The hair and scales are freed from fat by being placed for a few minutes in ether, washed with water, placed for one to two minutes in a 1 per cent. solution of silver, in order to kill the micro-organisms clinging to their surface, placed for a short time in sterile water and physiological salt solution, and again washed with water. The material to be examined (the hairs are cut into small particles) is distributed over the surface of suitable culture media. *Plauth*[1] recommends "cultivation *in situ*" as a diagnostic method, especially for trichophyta which grow at room-temperature, but also for the favus fungus, which develops only at higher temperatures.

Cultivation in Situ at Room-Temperature.—Several (three to four) hairs and scales are, without previous preparation and without treatment of the lesion, placed upon a sterile slide. They are crushed with a second sterile slide in order to spread them and make them sufficiently transparent for microscopical examination. They are then covered with a cover-glass, which is fastened at opposite sides with a drop of wax. The slide is now placed in a flat, moist chamber. This consists of a plate upon which is a glass dish, on which the slide is placed, and a glass bell, 12 centimetres in diameter and 7 centimetres in height. The interior of the bell is draped with filter-paper, which is fastened with drops of wax, and has an opening in the centre, through which the culture may be examined without removing the bell. After the slide and bell are in position, the plate is filled with water. Care must be taken that no water touches the culture.

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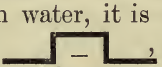
[1] *Zentralblatt f. Bakt. u. Parasitenkunde,* 1902, Bd. 31, No. 5.

If it is desired to transplant upon ordinary culture media, a small piece is cut from the edge of the scale, after the fungi are well developed, and transplanted upon maltose-agar, or used for making plate cultures according to *Král's* method.

In cultivating in an incubator, in order to protect the cover-glass from condensation water, it is covered with a bridge of moist filter-paper , which is fastened at the ends with wax and freshly moistened every morning.

The development of mycelium which in attempts at cultivation at room-temperature appears in the first two to three days, and that which spreads from the edge of the cover-glass and not from the hairs and scales, are to be considered as due to contamination. In cultivating in the incubator, contamination with fungi is easily recognized, since fructification takes place rapidly.

At room-temperature, trichophyta develop from the sixth to eleventh day onward. By cultivation in an incubator at 35° C. trichophytosis and favus can be diagnosed in this manner after only forty-eight hours.

Favus (Fig. 39)

The exciting cause of favus is the *Achorion Schoenleinii.* Its detection is easy as soon as the characteristic

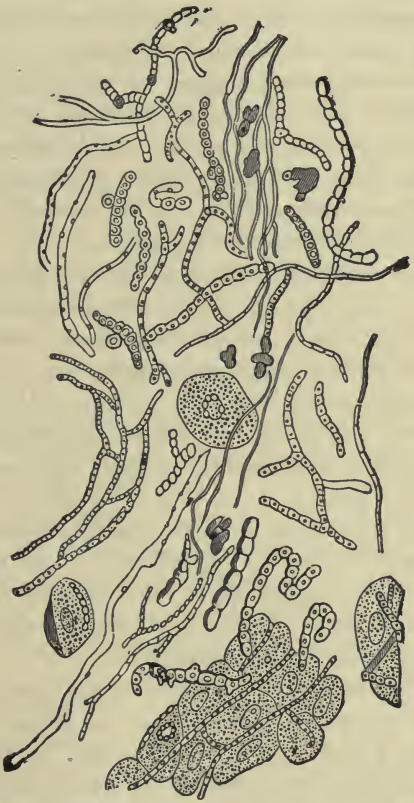


FIG. 39.—Favus Fungi, after *Lassar.*

crusts, the scutula, are present. These appear as compact, sulphur-yellow, cup-shaped bodies, which are usually pierced by a hair and embedded in the skin. They are isolated by piercing the horny layer, which at first covers them, and prizing them out of the skin. If the scutula do not appear distinctly, they can be made distinct by moistening the skin with alcohol. The specimens are examined unstained. The scutulum is seen to be composed of a finely granular mass, at the centre of which short, double-contoured, oval, round, or rectangular spores lie close together, and at the periphery of which radiating threads of mycelium are seen. These appear as threads of varying width, with many septa, often bifurcating and having bulbous ends. They also bud laterally and cut off the lateral hyphæ almost at a right angle.

The second important seat of favus fungi is the hair. Here, also, they may be clearly seen in unstained specimens. Longitudinal chains of mycelium, which are composed principally of rectangular members, are formed.

The fungi develop within the sheath of the root and in the hair itself; principally between the cuticula and the cortex, but also entering the cortex, as a rule, without splitting the hair.

The detection of the fungi in epidermal scales, in which they are usually present in but small numbers, is more difficult. They cannot, as a rule, be discovered in unstained specimens. *Bizzozero's* staining method (cf. p. 337) is best used for their detection.

The examination of the nails is also made by means of stained specimens. Threads of mycelium with spores are usually found. The favorite seat of the mycelial threads is between the bed and the lamina of the nail.

Cultural Procedures.—Favus fungi grow best at 35° C. upon culture media rich in nitrogen. After eight days the colonies are about the size of a pin's head, and after two to three weeks they are fully developed. The microscopical appearance of the cultures varies greatly, depending upon different factors, as the culture media, differences in temperature, age of the culture, etc. *Plauth* distinguishes between two main types: (1) The waxy type; yellowish spots of waxy consistency, which have radial folds and raised centres, and usually no air-mycelium, though they occasionally form a short down. (2) The downy type; white discs covered with a thick down, with irregular raised centres. The color varies; they may be snow-white, reddish, or yellow.

Animal Inoculation.—Gray mice are used, and contract favus when the material is rubbed into the skin at the base of the tail. A negative result of the test does not exclude favus, since not all favus fungi coming from man are pathogenic for mice.

Trichophytosis

Under the name of trichophytosis are included those diseases of the skin which are caused by fungi belonging to the group of the trichophyton. In spite of the numerous workers who have studied the etiology of these diseases, it has not as yet been definitely settled whether the different clinical manifestations of trichophytosis are caused by one and the same micro-organism, remarkable for its pleomorphism, or whether there are a variety of true trichophyta, to which the different clinical pictures owe their peculiarities.

Sabouraud,[1] especially, supports the latter view. He separates from the true trichophytosis a form of *Tinea tonsurans*, the so-called microsporia, which is caused by a small-spored fungus, the *Microsporon Audouini*

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[1] See "Regional Dermatology," by *Sabouraud*. Rebman Company, New York.

(Gruby), a variety of fungus which, according to his investigations, is entirely distinct from those exciting other forms of trichophytosis.

Microsporia.—Only the hairs are examined. The hairs, which protrude but slightly, break off when removed shortly above the surface of the scalp, the roots remaining in the matrix. They have a silvery-gray lustre, which examination with a magnifying-glass shows is due to a sheath surrounding the hair. Microscopical examination reveals that the sheath is almost entirely composed of small, closely placed ectospores. Within the air threads of mycelium, with peculiar gnarly, short branches, are seen. Cultivation is not necessary for diagnostic purposes, since the microscopical detection of the fungi in the hair is easy. Other forms of *Tinea tonsurans* are, according to *Sabouraud*, due to a large-spored fungus. The fungi appear within the hairs—which are thick, break off close to the scalp, and are difficult to remove—in the form of large round, somewhat irregular, distinctly double-contoured spores, which form long rosaries.

Tinea Sycosis (Fig. 40)

In the superficial form of *Tinea sycosis* the detection of the fungi is usually easy in the hair at the border of the rings. The spores lie, as a rule, about the follicles, the mycelium longitudinally within the inner sheath of the root, but, also penetrating the substance of the hair itself.

In the deeper form of sycosis *(Sycosis parasitica)* the microscopical detection of the fungus is more difficult. Its detection is easy, however, if cultures are made from the purulent secretion taken from the deepest portion of the lesion.

In order to tell which of a number of plucked hairs contain fungi, the hairs are moistened with chloroform; after the chloroform has evaporated the hairs containing

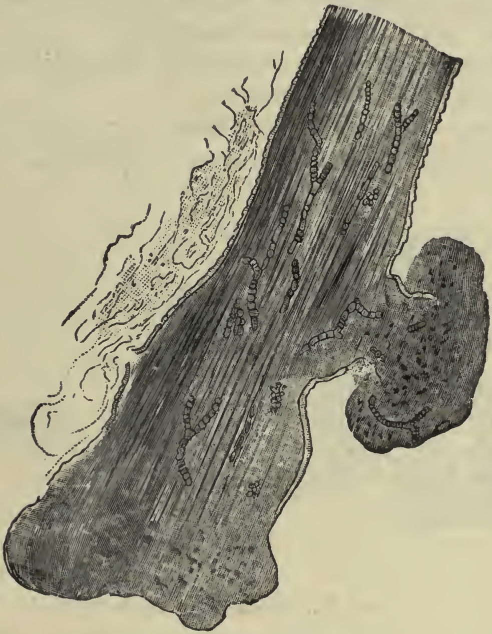

FIG. 40.—Hair in Sycosis.

fungi become chalky white. If the hairs are moistened with oil they regain their normal color.

Tinea Circinata (Fig. 41)

Trichophyta Circumscripta and Disseminata.[1]—The fungi appear in the epidermal scales as long, moderately branching threads, which give off but few conidia. They are, however, especially in *Trichophyta disseminata*, as a rule, so isolated that it is difficult to find them even in stained specimens.

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[1] See "Dermochromes." Rebman Company, New York.

Plauth recommends his method of cultivation *in situ*, as an aid in the diagnosis of these forms.

In *Eczema marginatum*, however, the fungi are present in great number in the scales.

In *Onychomycosis trichophytina* a luxuriant growth of spores is seen besides the threads of mycelium.

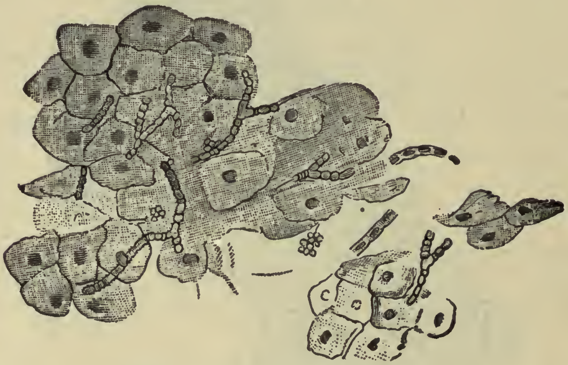


FIG. 41.—Epidermal Scales.

Cultural Behavior.—Trichophyta grow, in contrast to favus fungi, as well at 20° to 24° C. as at body-temperature, and flourish upon media poor in nitrogen, but rich in carbohydrates. Gelatine is liquefied. The cultures are remarkable for their great pleomorphism; the formation of pigment varies greatly in colonies from one and the same stock. On agar trichophyta form stars with many long rays radiating from a centre, which may be of varying appearance. It may be pyramidal, concave, or convex. The surface of the colony often appears as if powdered with flour, and occasionally a down of air-mycelium is formed. The colonies may be yellow, pink, violet, brownish-red, or brownish-black.

Differential Diagnosis.—It is only possible in a limited number of cases to differentiate between favus and trichophytosis by means of microscopical examination alone. If the typical favus products, the scutula, are present, microscopical examination reveals a very characteristic picture; but in just those cases in which the clinical diagnosis lies between favus and trichophytosis, as a rule, so few fungi can be detected that the points characteristic of favus fungi, in contrast to trichophyta—namely, their greater variety of form, their thicker, more gnarly threads, with numerous septa, and giving off branches more at a right than at an acute angle—are not sufficiently prominent in microscopical specimens to allow of a diagnosis.

Cultural procedures are also often of no aid in these cases, since undoubted reproductive organs, which ordinarily make it possible to distinguish the various types of hyphomycetes, are not known in the fungi of the skin; and the macroscopical appearance of the cultures differs so widely under the influence of various factors, that the cultures of favus fungi and trichophyta may resemble each other very closely. Finally, the results of animal inoculation are of diagnostic value only when positive. In many cases bacteriological examination is of diagnostic value only in so far as it may establish the fact that a dermatomycosis is present.

Pityriasis Versicolor

Pityriasis versicolor is excited by the *Microsporon furfur.* The micro-organism is present only in the horny layer of the skin. If the scales are examined in potassium hydrate, glycerine, or water, numerous fungi are seen, in the form of short U-shaped threads, with few branches, between which groups of spores are visible.

The microscopical picture is so characteristic that the use of cultural methods is not necessary for diagnostic purposes.

Cultivation of the fungi from the scales is very difficult. If, however, they have been cultivated, the following generations grow easily on the usual culture media, both at room- and at body-temperature. Before collecting the scales for cultivation, the skin is disinfected with bichloride of mercury washed with water, and sponged with a mixture of alcohol and ether. The scales are rubbed according to *Král's* method and planted upon urine agar (1 to 10) or *Fingers*' epidermin agar.

Erythrasma

Erythrasma is excited, according to the opinion of most authors, by the *Microsporon minutissimum.* This micro-organism also develops in the horny layer of the skin. The scales are best stained according to *Bizzozero's* method, and examined with the oil-immersion. The fungi are conspicuous for their exceptional delicacy. Long, winding threads, with many septa are seen, lying close together. Among the threads of mycelium numerous spores of varying shape are seen, which, because of their minuteness, may be mistaken for cocci.

Attempts to cultivate the fungus have not succeeded.

EXAMINATION FOR SPIROCHETA PALLIDA

The spirochetæ may be demonstrated in the smear, in the cut section, and in the fresh material. The animal experiment is not yet practical, nor have means been found to culture the spirochetæ.

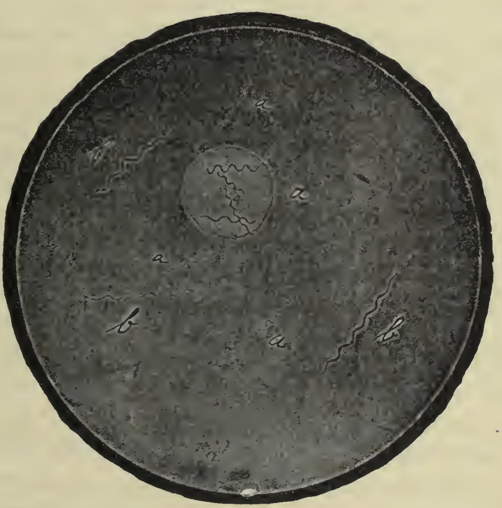


FIG. 42.—Spirochetæ from a Broad Condyloma, Magnified 1,500 Times; *a*, Spirocheta Pallida, *b*, Spirocheta Refringens.

Spirochetæ pallidæ have been cultivated to a number of generations, and animal experimentation successfully conducted at the Rockefeller Institute in New York. *Noguchi:* Journal of Experimental Medicine, Vol. XIV, No. 2, 1911, and Jour. Am. Med. Ass'n, July 8, 1911. *Noguchi* has obtained the spirochetæ pallidæ in pure culture, and he considers it as established that testicular lesions produced in rabbits by means of syphilitic materials are the result of the multiplication of the pallida and not of some associated indefinite parasite.

Method for Obtaining the Material for Observation

Chancres and eroded papules are first thoroughly cleaned with a piece of absorbent cotton which has been saturated with a physiological saline solution in order to remove the superficial secretions, which, as a rule, contain but few spirochetæ, but many other kinds of micro-organisms; then they are dried. Iodoform and other medications must first be removed, which is done by rubbing and applications of the physiological saline solution. The eroded surface is rubbed with a platinum loop until a slight serum (irritation serum) oozes out, which is used for the examination. A large admixture of blood is to be avoided. The secretion for the examination is easily obtained by milking the papule or the chancre with two fingers. Especially suitable for the examination is the scraping of the border of the erosion, which scraping has been done with a platinum spatula according to *Hoffmann.* A large number of spirochetæ is found in the secretions which were pressed out of the excised primary lesion.

In closed efflorescences the horny layer is removed with a knife, care being taken to avoid any hemorrhage, and the secretion is obtained from the border zone.

In pustules and pemphigus the blebs are first opened and the secretion obtained from the bottom.

Hoffmann's Method of Obtaining Material for Examination from Glands

The skin over the inguinal glands is first shaved, disinfected and washed with a physiological saline solution. A syringe of 5 cc is then used which has a long cannula and whose piston has asbestos packing. The syringe is first cleansed with a sterile saline solution. The gland is then held with the left hand and the cannula inserted into it. We carefully attempt to introduce the aspirating needle into the substance (*Rindenschicht*) of the largest gland (in the direction of the long diameter) and then begin to aspirate, gradually removing the point of the needle. If we don't obtain enough of secretion, the needle is forced into a neighboring gland or several glands until we get a few light red drops. We know that the needle is in the gland, if we move the gland and the needle follows the motion. The substance so obtained from the gland is squirted into a small sterile dish; the single droplets are thoroughly mixed together. If necessary, the dishes are covered, and then thin smears are prepared as rapidly as possible.

Blood for the purpose of examination is obtained by puncturing either the vein or the ear. At least 1 cc of blood is removed and mixed with ten times its quantity of ½ per cent. solution of acetic acid. The dissolved blood is centrifuged and the sediment examined.

The Preparation of Stained Specimens

For the preparation of smears we use either cover-glasses or slides which have been kept for several days in a solution of equal parts of ether and alcohol; these are then most carefully cleaned. A drop of the serum to be examined is taken up with the margin of a cover-glass which is then rapidly moved upon the slide from left to right, the cover-glass being kept inclined. In this way we get a thin smear of equal thickness, which is fixed by immersion in absolute alcohol for ten minutes and osmic acid vapors. The latter may be performed in the fixation tubes of *Hamm*. One end of this tube is filled with glass wool which is saturated with a solution of 1 per cent.

osmic acid in a solution of 1 per cent. chromic acid. The slide is first put into the tube for one to two minutes, after which the smear is prepared, and the wet smear is exposed to the fumes of the osmic acid for twenty to forty seconds; it is then dried in the air, after which it is passed three times through the flame.

The fixation with the osmic acid fumes can be done also in this way: Into a double dish is put an open glass dish of 5 cm diameter, into which are put 5 cc of a 1 per cent. solution of osmic acid and 10 drops of glacial acetic acid. The slide is put into this glass dish before the smear is made and exposed to the osmic acid fumes for two minutes. The smear is then quickly made over the surface which was exposed to the osmic acid, and the slide, still wet, again exposed to the fumes of the osmic acid for another one to two minutes. After the specimen has dried in the air, it is put into a very light red solution of permanganate of potash for one minute, then washed with water and dried between filter-paper.

Method of Staining

There are a great many methods, but the best is with *Giemsa* solution manufactured either by *Gruebler* of Leipzig, or by *Leitz* of Berlin.

Preparation of the Staining Fluid

Ten drops of *Giemsa* solution are shaken up in a glass beaker with 10 cc of distilled water which is free from acids. Care must be taken that the staining pigment is not precipitated while shaking. This solution must be prepared freshly before use.

The Staining

The specimen is put into a flat glass dish, the smear downward and the staining solution is poured over it

The cover-glasses are kept on glass rods. The duration of staining is one to two hours. Before the specimen is removed from the staining solution, a thin membrane which formed on the surface, is first removed with filter-paper. The specimen is then washed with water and dried between filter-paper.

Method for Quick Staining

The specimen is covered with the diluted *Giemsa* solution and held over a flame until it steams. After a quarter of a minute the staining fluid is poured off. This procedure is repeated four times, but at the fourth time the staining fluid remains on for one full minute. Thereupon the specimen is washed with water and dried with filter-paper. In a well-stained specimen with *Giemsa* solution the spirochetæ are stained a distinct red and the leucocytes a very dark red; in unsuccessful staining the coloring appears blue. Since the spirochetæ are found mostly in the neighborhood of the red blood-cells, we look under the microscope for these first. For the examination oil immersion is used and a strong ocular (compensation ocular 4).

Examination of the Fresh Specimen

A small drop of the material to be examined is put on a cover-glass, a little of a physiological salt solution is added, the cover-glass is then put on a slide and the margins of the cover-glass are smeared with vaseline or wax. Hanging-drop examinations can also be made, but the drop must be as flat as possible. Apochromatic and compensation ocular 6 to 12 are used. Strong illumination is necessary. The spirochetæ are found more easily in the dark field illumination.

Examination of Cut Sections

The old method of *Levaditi* is the best.

The slices to be imbedded should be no thicker than 2 mm. All apparatus to be used must be scrupulously clean.

1. Fixation for twenty-four hours in a 10 per cent. solution of formalin (longer fixation does not hurt).

2. Hardening for about twelve hours in 95 per cent. alcohol.

3. Washing repeatedly in distilled water until the pieces sink to the bottom.

4. Impregnation with ½ to 3 per cent. silver nitrate solution which is contained in a wide-mouthed 100 cc flask and left in the incubator at 35° to 37° C. for three to five days. It is best to renew the silver solution every day.

5. Reduction of the pieces remaining in the flask, after the silver solution has been poured off with the following solution:

Pyrogallol . . .	4.0
Formalin	5.0
Aq. dest.	100

In this solution the pieces remain for forty-eight hours at room-temperature. This solution must be prepared freshly every time and renewed daily.

6. Washing with distilled water.

7. Hardening in alcohol the strength of which has been gradually increased, and imbedding in paraffin. The slices must not be any thicker than 5 to 10 micromillimetres. After-staining is not necessary.

The spirochetæ appear very dark, almost black, the tissues are yellow.

Other Methods of Staining Spirochetae Pallidae (Spirochetes: Bosanquet)

Davidsohn recommends the use of cresyl violet. *Oppenheimer* and *Sachs* use carbolic gentian violet (concentrated alcoholic gentian violet solution, 10 cc, solution of phenol, 5 per cent., 100 cc. *Proca* and *Vasilescu* use *Gino de Rossi's* cilia stain (dissolve fifty gm. pure phenol and forty gr. tannin in 100 cc water and add to this 2.5 gm. basic fuchsin dissolved in 100 cc absolute alcohol. Stain in this for ten minutes, wash and dry. Then stain with a mixture containing concentrated alcoholic gentian violet, 10 cc; phenol, five gm.; distilled water, 100 cc.

Reitmann advises that films should be first treated with a 5-per-cent. solution of phosphoric acid in water for five minutes and then stained with carbol-fuchsin, warmed.

Goldhorn uses a complicated preparation of polychrome methylene blue (methylene blue, lithium carbonate and eosin, and *McNeal* also uses methylene blue and eosin (crude methylene blue, twenty parts; pure medicinal methylene blue, ten parts; eosin (yellowish), twenty parts; and pure methyl alcohol, 100 parts. Stain on cover-slip for forty-five to sixty seconds; wash in dilute solution of sodium carbonate, one drop of 1-per-cent. solution in 10 cc water).

A special method of staining with India ink is suggested by *Burri.* For this purpose, ordinary India ink is diluted with water (one part in six, or one in ten). The solution is sterilized and allowed to stand for two weeks, the supernatant fluid being then ready for use. A loopful of suspension of the organisms is mixed with a drop of the ink-solution, spread on a slide and allowed to dry. The spirochetæ are then easily seen as colorless spirals on a dark background. Some writers prefer a stronger solution of the ink, i.e., one part in two of water.

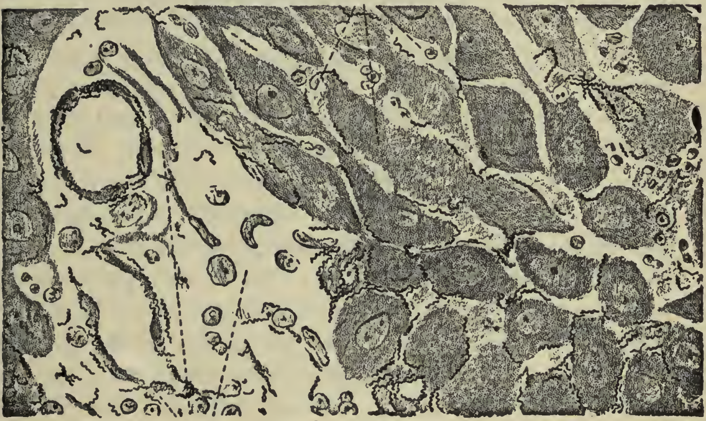
Mandelbaum stained living spirochetæ pallidæ in a hanging drop by adding a loopful of *Loeffler's* methylene blue solution along with a loopful of decinormal soda solution. *Meirowsky* makes a paste of methyl violet and salt solution, and rubs it into the previously cleaned surface of the chancre. In the serum which exudes, there are found stained specimens of spirocheta pallida and spirocheta refringens. Certain more deeply staining dots in the substance of the organisms he regards as nuclei. Crystal violet is as efficacious as methyl violet.

Morphology

In the specimen which has been well stained with *Giemsa* solution, the spirochetæ appear as very fine red-stained spirals, the ends of which are mostly very finely pointed. They show from 6 to 20 spirals the windings of which are at regular intervals. Notwithstanding the numerous winding some spirochetæ appear almost like a straight line. As a rule, the spirochetæ are found singly, often they are found also in twos, forming an acute angle or the one rapidly running after the other, or they are grouped in the form of a ''Y''; occasionally they are in heaps, balls or twisted in the form of tresses. In the same specimen may be found typical and atypical forms, the windings of which are in some very short, and in some the windings are not noticeable at all, so that they appear like thin threads. The ends of some spirochetæ are coiled in the form of a spiral and in others they are club-shaped. These may possibly be artifices which were produced in the preparation of the specimen.

The characteristics of the living spirochetæ are: they are very fine, have little refractive power, their numerous windings are regular, narrow, deep and almost straight, they do not change their form in motion or when at rest. This constancy of form gives to the spirochetæ the peculiar appearance of having been turned on a lathe. Toward the end the windings are not so high, the ends as a rule being pointed. The same specimen may show long and short spirochetæ, whose motion is within a short radius. We observe rotatory motions on their long axis, we see them move forward and backward, we see their bodies flex upon themselves which may be compared with the bending and the straightening out of an elastic tube.

Epithelium with migrating cells.



Papillary bodies with vesicles.

FIG. 43. After *Blaschko.*

Differential Diagnosis

In the differential diagnosis we have to consider the spirocheta refringens, ballantidis, buccalis, Vincenti, dentium and the sp. pallidula pertenuis. But the sp. pallida may be distinguished from these after a little careful study. The one exception makes the sp. pallidula found in frambœsia tropica which, so far, could not be differentiated morphologically from the sp. pallida.

In a specimen stained with *Giemsa* solution the sp. pallida is red, but mostly the rest of the spirochetæ are from a violet to a blue color. The other varieties are mostly thicker and fatter as compared with their length, while the finer forms are shorter than the sp. pallida. They do not end in fine points as the sp. pallida, and their windings are flatter and irregular. In the fresh specimen their motility is much greater; they are more refractive and therefore more easily found than the sp. pallida. Furthermore, they do not show such constancy of form as the sp. pallida; they show their windings only while in motion, and when at rest they straighten out more or less, showing nearly a straight line. The sp. dentium shows very great similarity to the sp. pallida, as it also stains red with *Giemsa;* is also very fine, has regular windings, has little refractive power, and does not change its form in motion. But they are to be differentiated from the sp. pallida, because of their spirals not being so deep as in the sp. pallida.

In order to avoid mistakes only such spirochetæ should be taken into consideration which correspond in all respects with the normal type, when we are to make the diagnosis from the morphological properties alone.

CHAPTER XII

THE USUAL METHODS OF BACTERIOLOGICAL EXAMINATION, FORMULAE OF STAINS, AND CULTURE MEDIA

I. Examination in a Hanging-Drop

For the examination in a hanging-drop a concave slide is used. A layer of vaseline is smeared around the margin of the concavity. A drop of sterile, physiological (0.85 per cent.), sodium-chloride solution, or bouillon, is placed with a sterilized platinum wire in the centre of a cover-glass, which is held in a *Cornet* forceps, and a very small quantity of the material containing the bacteria is placed in it by means of a sterile wire. If the material is fluid, a drop of it is placed directly upon the cover-glass. The drop should be flat and round. The cover-glass is so placed upon the slide that the drop hangs free in the concavity, which is completely closed by pressing the cover-glass firmly against the vaseline.

In the microscopical examination the concave mirror and the iris diaphragm are used. First, the low power and a very narrow diaphragm are used, and the margin of the drop is so placed that it crosses the centre of the field as a bright line. The diaphragm is then somewhat opened, a drop of cedar-oil placed upon the cover-glass, without shifting the specimen, and the low power replaced by the oil-immersion. The margin of the drop is again brought into focus. The lens must be carefully lowered, in order to avoid shattering the cover-glass.

II. Examination in Stained Smears

1. Preparation of the Specimens

1. The material is smeared upon cover-glasses held in *Cornet* forceps. Fluid material is spread, directly, in an even, thin layer over the entire surface of the cover-glass by means of a platinum wire; solid material after being mixed with a drop of sterile water.

2. The smear is dried in the air. Drying may be hastened by carefully warming the cover-glass over the flame, with the smeared side up.

3. Fixation.

The cover-glass, with the smeared side up, is passed through the flame three times. For special purposes—for example, examination of blood—the smears are fixed by placing in alcohol (ten minutes), or in alcohol and ether āā (two to ten minutes). For *Sobernheim's* method of fixation, cf. p. 54.

4. Staining.

As much stain as will remain upon it without overflowing is dropped from a pipette or a dropping-bottle upon the cover-glass, which is held in a pair of *Cornet* forceps.

The stain is allowed to act at ordinary temperature, or is heated to the steaming-point over a small flame. The length of staining varies from a few seconds to several minutes, depending upon the variety of bacteria and the method of staining.

5. Wash with water.

6. Dry with filter-paper.

7. Mount in Canada balsam.

Stained specimens are examined with the oil-immersion, with wide diaphragm, and the plane-mirror. Low-power oculars are always used in examining the specimens, since with high-power oculars the objects, though larger, are darker and less distinct.

2. Staining Methods and Staining Solutions

Bacteria are stained with basic aniline dyes. Those most frequently used are fuchsin, methylene blue, Bismarck brown, methyl violet, dahlia, and gentian violet. Most bacteria, with the exception of the acid-fast, stain with dilute watery solutions. As these, however, keep but a limited time, stock solutions are made, which can be kept a long while, and diluted each time for use. All staining solutions must be carefully filtered.

Stock Solutions

Saturated alcoholic solutions of fuchsin, methylene blue, and gentian violet, are made by placing sufficient dye in a glass-stoppered bottle of alcohol so that a portion remains undissolved. The solution is filtered from the precipitate. *Ziehl's* or *Czaplewski's* carbol-fuchsin is often used as stock solution for fuchsin, borax-methylene blue as stock solution for methylene blue.

Ziehl's Carbol-Fuchsin		*Czaplewski's Carbol-Fuchsin*	
Fuchsin . . .	1.0	Fuchsin . . .	1.0
Alcohol . . .	10.0	Acid. carb. liquefact.	5.0
Acid. carb. liquefact.	5.0	Glycerine . . .	50.0
Aqua dest. . . .	100.0	Aqua dest. . . .	100.0

Borax-Methylene Blue

Methylene blue	2.0
Borax	5.0
Aqua dest.	100.0

The saturated alcoholic stock solutions are diluted before using with distilled water, in a test-tube, until they are just transparent.

The dilute solutions of carbol-fuchsin and borax-methylene blue are made by diluting with ten times the volume of distilled water.

GRAM'S METHOD

1. Carbol-gentian violet, three minutes, without heating.
2. *Lugol's* solution, one minute and a half.
3. Ten per cent. acetone-alcohol as long as clouds of stain are given off from the smear.
4. Wash with water.
5. Bismarck brown, one minute, or carbol-fuchsin in a dilution of 1:20 aqua dest., five seconds.
6. Wash with water, dry, etc.

After (1) and (2) the stain is poured off (do not wash with water) and the smear dried with filter-paper.

Carbol-Gentian Violet

Gentian violet . .	1.0
Alcohol . . .	10.0
Acid. carb. liquefact.	5.0
Aqua dest. . . .	100.0

Acetone Alcohol

Acetone . . .	10.0
Alcohol abs. .	ad 100.0

Lugol's Solution

Iodine	1.0
Potassium iodide .	2.0
Aqua dest. . . .	300.0

Bismarck Brown

Bismarck brown . .	1.0
Alcohol . . .	10.0
Aqua dest. . . .	100.0

STAINING OF TUBERCLE BACILLI AND OTHER ACID-FAST BACILLI

(*a*) *Method of Ziehl-Neelson*

1. Carbol-fuchsin three minutes, heating to the steaming-point.
2. Wash with water.

3. Twenty per cent. nitric acid, three to five seconds.
4. Wash with water.
5. Decolorize with 60 per cent. alcohol.
6. Wash with water.
7. Dilute methylene-blue solution one minute.
8. Wash with water, etc.

Carbol-fuchsin, cf. p. 329

(*b*) *Czaplewski's Method*

1. Carbol-fuchsin, heating to the steaming-point.
2. Pour off stain, but do not wash with water.
3. Dip in fluorescin-methylene blue six to ten times.
4. Dip in a concentrated alcoholic solution of methylene blue ten to twelve times.

If necessary, repeat 3 and 4.

5. Wash with water, etc.

Fluorescin-Methylene Blue

Yellow fluorescin (*Gruebler*)	1.0
Alcohol	100.0
Allow to stand one to two days, decant from precipitate and add methylene blue	5.0

Shake; allow to stand one day and decant from precipitate.

Concentrated Alcoholic Solution of Methylene Blue

Methylene blue	5.0
Alcohol	100.0

Filter before using.

(*c*) *Method of Fraenkel and Gabbet*

1. Stain with carbol-fuchsin for three minutes, with the aid of heat.

2. Simultaneous decolorization and counter-staining with the following mixture:

Saturated alcoholic solution of methylene blue	50.0
Sulphuric acid	25.0
Aqua dest.	100.0

(*d*) *Pappenheim's Method*

(Differential stain between tubercle bacilli and other acid-fast bacilli:)

1. Stain with carbol-fuchsin for three minutes, with the aid of heat.

2. Dip three to five times in the corallin solution, without previous washing with water.

3. Wash with water, etc.

Corallin

Corallin	1.0
Saturated alcoholic solution of methylene blue	100.0
Glycerine	20.0

(*e*) *Baumgarten's Method for Differentiating Lepra Bacilli*

1. Stain with very dilute carbol-fuchsin for five minutes.

2. Decolorize with a solution of 1.0 nitric acid in 10.0 alcohol for twenty seconds.

3. Wash with water.

4. Counter-stain with methylene blue.

STAINING OF DIPHTHERIA BACILLI

(*a*) Stain with carbol-fuchsin 1 in 10 aqua dest. for one minute, without heating.

(*b*) Stain with *Loeffler's* alkaline methylene blue for two minutes, without heating.

Loeffler's Alkaline Methylene Blue

Concentrated alcoholic solution of methylene blue	30.0
0.01 per cent. watery solution of potassium hydrate	100.0

(*c*) Stain according to *Roux* for two minutes, without heating:

1. Dahlia violet	1.0	2. Methyl green	1.0
Alcohol	10.0	Alcohol	10.0
Aqua dest.	100.0	Aqua dest.	100.0

For use one part of stain 1 is mixed with two parts of stain 2. This solution may be kept on hand.

(*d*) *Neisser's* stain.

1. Stain 1 for twenty to thirty seconds.
2. Wash with distilled water.
3. Stain 2 for ten to fifteen seconds.
4. Wash with distilled water, etc.

1. Methylene blue	1.0	2. Bismarck brown	2.0
Alcohol	20.0	Aqua dest.	ad 1000.0
Acid. acet. glacial	50.0		
Aqua dest.	ad 1000.0	(Decomposes easily.)	

New Method

1. Stain 1 fifteen to thirty minutes.
2. Washing with water.
3. Stain 2 fifteen to thirty minutes.
4. Washing with water.

Stain 1 consists of 2 parts of solution a and 1 part of solution b.

Solution a.		*Solution b.*	
Methylene blue	1.0	Crystal violet	1.0
Alcohol	20.0	Alcohol	10.0
Aqua dest.	1000.0	Aqua dest.	300.0
Aqua acet. glacial	50.0		

Stain 2, Chrysoidin, 1.0; Aqua dest. fervid. 300.0.

STAINING OF GONOCOCCI

(*a*) Stain with very dilute methylene-blue solution for two minutes, without heating.

(*b*) Stain according to *Gram*, cf. p. 330.

(*c*) Double staining methods.

Pappenheim's Method (*Krystallowicz's Modification*).

Stain for one minute with the following solution, without heating:

Methyl green	0.15
Pyronin	0.25
Alcohol	2.5
Glycerine	20.0
Aqua carbolisat. 2 per cent. ad	100.0

May and Gruenwald's Method

Stain for two minutes. The cover-glass is placed in the solution unfixed, and with the smeared side down. For the formula of the stain, cf. p. 258.

STAINING OF SPORES

Klein's Method

1. An agar culture which contains spores is floated in physiological salt solution, the mixture treated with an equal quantity of carbol-fuchsin, slightly heated, and set aside for about half an hour.
2. Smears are made from the mixture, allowed to dry in the air, and fixed in the flame.
3. Decolorize in 1 per cent. sulphuric acid for one to two seconds.
4. Wash with water.

5. Stain with dilute methylene blue for three to four minutes.

The spores are stained red, the bacilli blue.

Staining of the Capsules of Anthrax Bacilli

(*a*) *Johne's Method*

1. Stain with a 2 per cent. watery solution of gentian violet for two minutes, heating carefully.
2. Wash with water.
3. Decolorize with 1 to 2 per cent. acetic acid for six to ten seconds.
4. Wash with water.

Examine in water, not in Canada balsam.

(*b*) *Raebiger's Method*

1. Stain with formalin-gentian violet for twenty seconds without previous fixation.
2. Wash with water, dry, and mount in Canada balsam.

Formalin-gentian violet.

Fifteen to twenty grammes of gentian violet are covered with 100 to 200 cc of formalin, the mixture thoroughly stirred, allowed to stand several hours, and filtered.

(*c*) *Hamm's Method*

1. Fixation in osmium fumes (cf. p. 320).
2. Spreading of the bacteria in ascitic fluid. The material is carefully rubbed up with a drop of ascitic fluid in spiral motions. Staining ten to fifteen minutes with a dilute *Giemsa* solution (10 drops to 10 cc aqua dest.), warming it slightly the last three to five minutes.

Staining of Flagella

For the demonstration of flagella a very dilute mixture of bacteria is made from a young agar culture, and spread in a very thin smear upon cover-glasses which are absolutely clean and free from fat.

In order to avoid overheating, the smear is held in the fingers when passed through the flame for fixation.

(*a*) *Loeffler's Method*

1. Fix the flagella, heating just to the steaming-point. (For the mordant, see below.)
2. Wash with water until the mordant is entirely removed.
3. Wash with alcohol.
4. Stain with aniline-water-fuchsin solution, to which 1 per cent. sodium hydrate has been added until precipitation commences, one minute, heating to the steaming-point.
5. Wash with water, dry, mount in Canada balsam.

Mordant

Twenty per cent. solution of tannin, 10 cc.
Cold saturated solution of ferrous sulphate, 5 cc.
Watery or alcoholic solution of fuchsin, 1 cc.

For some bacteria alkali (a few drops of a 1 per cent. solution of NaOH) must be added to the mordant; for other acids (H_2SO_4).

Aniline Water

Five parts of aniline oil are added to 100 parts of water, the mixture shaken thoroughly, and filtered through a moist filter. The filtrate must be absolutely clear. The dye is either dissolved in the aniline water directly, or sufficient of a concentrated alcoholic solution of the dye is added to the aniline water to produce a distinct opalescence.

(*b*) *Bunge's Method*

1. Fix the flagella for one to five minutes with the aid of heat.
2. Wash with water.
3. Dry between filter-paper.
4. Stain with carbol-gentian violet with the aid of heat.
5. Wash with water, etc.

Mordant.—Three parts of a concentrated watery solution of tannin are mixed with 1 part of a 1 to 20 watery solution of liquor ferri sesquichlor.; 1 cc of a concentrated watery solution of fuchsin is added to 10 cc of this mixture.

The mordant must stand several days. Each time before using H_2O_2 is added, a drop at a time, until the solution is reddish-brown.

Other methods have been suggested by *Van Ermengen*, *Zettnow*, and others.

STAINING OF FUNGI

(*a*) *Bizzozero's Method, modified by Plauth*

"Scales are placed in glacial acetic acid on a slide and crushed with a second slide. Harden and dehydrate with alcohol; heat until the alcohol and acetic acid have evaporated and the scales, still somewhat moist, lie upon the dry slide. Stain with *Ziehl's* solution for three minutes. Remove the solution carefully with a piece of filter-paper. Iodine-potassium iodide solution (1:2:300) for one minute. Decolorize with aniline oil until no more clouds of stain are given off. Examine in aniline or xylol. The fungi appear dark red, the tissue pale pink." (*Plauth* in "Handbuch d. pathog. Mikro-org. v. *Kolle* und *Wassermann*.")

(*b*) *Waelsch's Method*

1. Mixture of aniline water (cf. p. 336) and a concentrated alcoholic solution of gentian violet (2:1) for ten to fifteen minutes.

2. Mixture of equal parts H_2O_2 and 5 per cent. watery solution of potassium iodide, three minutes.

3. Decolorize completely with aniline oil, to which 1 per cent. HCl has been added (thick scales, nails, and hair, eight to ten hours; thin scales and microtome sections, two to six hours).

4. Wash in xylol.

5. Mount in Canada balsam.

(Microtome sections may be previously stained with picrocarmin.)

(*c*) *Kuehne and Weigert's Method*

1. Crystal violet (cf. p. 342) about five minutes.

2. *Lugol's* solution until stained black (one to two minutes).

3. Dry with filter-paper.

4. Aniline oil until no more dye is given off.

5. Xylol (to remove the aniline oil).

6. Canada balsam.

Staining of Blood Specimens

(*a*) *Manson's Method*

1. Stain with borax-methylene blue (cf. p. 329), which has been diluted until just transparent when examined in a test-tube, five to ten seconds.

(The specimen is dipped into the staining solution.)

2. Wash in a glass of ordinary water until the specimen shows a greenish tinge.

3. Dry, and mount in cedar-oil.

(*b*) *May and Gruenwald's Method* (cf. p. 258)

Stain two minutes. The cover-glass is placed in the staining solution with the smeared surface down.

(*c*) *Giemsa's Method* (*a Modification of Romanowski's Method*)

Stock solutions: 1 per cent. watery solution of eosin, 0.08 per cent. watery solution of azur *(Hoechst)*.

Preparation of staining solutions: 1 cc of the 1 per cent. eosin solution is added to 200 cc of aqua dest. To 9 cc of this solution 1 cc of the 0.08 per cent. azur solution is added.

The specimen is floated upon this mixture in a watch-glass. The length of staining varies from ten minutes to several hours. The staining is controlled with the microscope by examining the smear mounted in water (with the dry system). On the appearance of precipitation of the stain the smear is washed with 30 to 40 per cent. alcohol.

III. Examination of Cut Sections

The pieces of tissue are hardened in alcohol.

Embedding in Paraffin

1. Place in aniline oil until the specimen is transparent. (Place in a closed glass in the paraffin oven.)

2. Place in xylol, which is repeatedly changed, until the xylol no longer turns yellow (about one hour).

3. Dry with filter-paper.

4. Place in fluid paraffin (melting-point, 56° C.) in a

thermostat at 54° C. The paraffin is changed once. Stay in the thermostat one to four hours, according to the size of the specimen.

5. The specimen and paraffin are placed in an embedding frame. The paraffin is quickly solidified by covering it with water or placing it in an ice-chest.

The block of paraffin is suitably cut, fastened upon a block of wood by melting slightly, and cut with a microtome with a dry knife.

The individual sections are taken from the knife and placed directly upon a slide, which has been smeared with glycerine-albumin, and moistened with water. The water is poured off, the remainder absorbed with filter-paper, and the slide placed in an incubator. After twelve hours the sections are treated in the following manner:

1. Remove the paraffin by placing in xylol.
2. Place in absolute alcohol.
3. Place in 96 per cent. alcohol.
4. Place in water.
5. Stain.
6. Wash in water.
7. Dehydrate in alcohol.
8. Clear in xylol.
9. Mount in Canada balsam.

Glycerine-Albumin Solution

A measured quantity of egg-albumin is beaten to a froth, an equal quantity of pure glycerine added, and the mixture filtered.

EMBEDDING IN CELLOIDIN

Two solutions of celloidin are made in alcohol and ether āā, a thin solution and a thick, syrupy solution.

The specimens, which should not be thicker than 1 centimetre, are taken from the absolute alcohol, and placed for at least twenty-four hours in the thin solution of celloidin, and then for the same length of time in the thick solution. They are then placed on a cork, gradually covered with the thick solution, and, in order to prevent too rapid evaporation, covered with a glass bell. When the celloidin is sufficiently dry, the specimens are placed for twenty-four hours in 80 per cent. alcohol.

When cutting the specimens, the knife and specimens are moistened with alcohol.

Further Treatment of the Sections

1. Place in dilute alcohol.
2. Stain.
3. Dehydrate in 96 per cent., then in absolute alcohol.
4. Clear in xylol.
5. Mount in Canada balsam.

Universal Staining Methods for Demonstrating Bacteria in Sections

Loeffler's Method

1. Stain in *Loeffler's* methylene blue three to five minutes.

2. Differentiate in 0.5 to 1 per cent. acetic acid ten to twenty seconds.

3. Dehydrate in alcohol, xylol, Canada balsam.

Staining with Gentian Violet

1. Stain in a 2 per cent. watery or alcoholic solution of gentian violet until the sections are dark violet.

2. Wash in absolute alcohol until the sections are light violet.

3. Clear in xylol, and mount in Canada balsam.

Pfeiffer's Method

1. Stain in carbol-fuchsin (1:10) thirty minutes.

2. Differentiate in 60 per cent. alcohol, to which 1 drop of acetic acid has been added, until the sections are grayish-violet.

3. Dehydrate in absolute alcohol, xylol, Canada balsam.

SPECIAL STAINING METHODS

Gram's Method

1. Stain with aniline water gentian violet (cf. p. 336) five to thirty minutes.

2. *Lugol's* solution (cf. p. 330) one to two minutes.

3. Differentiate in absolute alcohol until the sections are nearly colorless.

4. Wash in water.

5. Stain with Bismarck brown (cf. p. 330) one to two minutes.

6. Place in 60 per cent., then in absolute alcohol, xylol, Canada balsam.

Kuehne and Weigert's Method

1. Stain in lithium carmin two to three minutes.

2. Wash in 3 per cent. HCl alcohol (70 per cent.).

3. Wash in aqua dest.

4. Stain with crystal violet five to ten minutes.

5. Treat with *Lugol's* solution until the sections become black (about one to two minutes).

6. Dry with filter-paper.

7. Treat with aniline oil until no more of the dye is given off.

8. Clear with xylol, and mount in Canada balsam.

Lithium Carmin

Carmin, 2.5 to 5.0; saturated watery solution of lithium carbonate, 100.0.

Crystal Violet

Stock solution: Crystal violet, 1.0; alcohol, 10.0.

Staining solution: One cc of stock solution is diluted with 10 cc of aqua dest., and treated with 1 drop of HCl.

STAINING OF TUBERCLE BACILLI

(*a*) 1. Stain with carbol-fuchsin thirty minutes (in incubator at 37° C.).

2. Wash with water.

3. Decolorize in 3 per cent. HCl alcohol (70 per cent.).

4. Wash with water.

5. Counterstain with dilute methylene blue two to three minutes.

6. Wash in water.

7. Alcohol, xylol, Canada balsam.

(*b*) 1. Stain in carbol-fuchsin thirty minutes.

2. Decolorize in 20 per cent. nitric ten seconds, and 60 per cent. alcohol until the sections are colorless.

3. Wash in water.

4. Counterstain with dilute methylene blue two to three minutes.

5. Wash with water.

6. Alcohol, xylol, Canada balsam.

STAINING OF DUCREY'S BACILLI

(*a*) *Peterson's Method for Paraffin Sections*

1. Stain in *Unna's* methylene-blue solution twenty-four hours.

2. Aniline oil about three to four hours.
3. Aniline xylol one and a half to three hours.
4. Xylol, Canada balsam.

(*b*) *Krefting's Method for Celloidin Sections*

1. Stain on the slide in *Unna's* methylene blue two to five minutes.
2. Dry with filter-paper.
3. Aniline xylol two to three hours.
4. Xylol, Canada balsam.

Unna's Methylene Blue

Methylene blue,		
Potass. carbon	āā	1.0
Aqua dest.		100.0
Alcohol		20.0
M. coque ad reman. 100.0. Adde		
Methylene blue,		
Borax	āā	1.0
In aqua dest.		100.0

Soluta misce.

IV. Cultural Methods

Preparation of Culture Media

Potato

The potatoes are cleansed with a brush in running water, the eyes cut out, peeled, sliced, placed in Petri dishes, and steam-sterilized for one hour on three successive days.

Cylinders may be cut from the peeled potatoes with a wide cork-borer, and divided into halves by an oblique cut. The wedges of potato so obtained are placed with the base down in broad test-tubes, which have a constriction about 1 centimetre above the tip (*Roux's* tubes), and sterilized in the above manner. Instead of *Roux's* tubes ordinary test-tubes may also be used, in whose tip a little cotton is placed to absorb the condensation water.

The potatoes may be rendered surely alkaline by boiling ten minutes in soda solution.

NUTRIENT BOUILLON

1. Lean chopped meat is covered with twice its quantity of water.

2. One per cent. peptone and 1 to 2 per cent. sodium chloride (calculated according to the quantity of water) are added.

3. Boil in steam-sterilizer one to two hours per litre of fluid.

4. Filter through a moist folded filter.

5. Neutralize with a saturated soda solution or 25 per cent. sodium hydrate until blue litmus-paper is no longer turned red, while red is turned slightly blue.

6. Boil in steam-sterilizer for one-half to one hour per litre of fluid.

7. Filter. The filtrate must be absolutely clear.

8. Test the reaction. If this must be corrected it is necessary to again boil and filter.

9. Pour into test-tubes which are closed with cotton plugs and have been sterilized by dry heat for half an hour at 160° C.

10. Sterilize in steam-sterilizer for half an hour on three successive days. During the interval keep at room-temperature.

NUTRIENT AGAR

1 to 8. As in preparing nutrient bouillon.

9. Add 2 per cent. finely cut or pulverized agar; to dissolve, boil for three to five hours per litre of fluid.

10. Clear by adding the white of an egg, which has been stirred in 50 cc of water, to the culture medium, which has been cooled to 50° C.

11. Boil for two hours per litre.

12. Filter in steam-sterilizer (cover the funnel carefully with filter-paper).

13. Pour into sterile tubes in quantities of 15 cc per tube (full tubes), which are later used for making plates, and in quantities of 5 cc for making slanting agar tubes.

14. Sterilize as in preparing nutrient bouillon.

For the filtration of the agar the filter is prepared in the following way :[1] A piece of absorbent cotton is put into the angle of an enamel or other funnel which will stand heat, and over this a closely woven piece of wire netting. A similar piece of wire netting is placed over the opening of the funnel; over this a thin sheet of absorbent cotton and another piece of wire netting over the cotton. With a filter so constructed the filtration can be accomplished in a very short time after the sediment has well settled on the bottom of the hot kettle, whilst the agar was clarifying.

NUTRIENT GELATINE

1 to 4. As in preparing nutrient bouillon.

5. Add 10 to 15 per cent. (in summer) gelatine.

6. Dissolve by slight heating.

7. Neutralize (cf. Nutrient Bouillon, § 5).

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[1] After the description of a laboratory worker in *Erbacher's* Institute for Medical Diagnosis.

8. Clear (cf. Nutrient Agar, § 10).
9. Boil for three-quarters of an hour.
10. Test the reaction.
11. Filter in hot-water funnel.
12. Pour into tubes.
13. Sterilize in steam-sterilizer for a quarter of an hour on three successive days.

After sterilization solidify at once by placing in ice-chest, then keep at room-temperature.

In preparing the culture media 1 per cent. of Liebig's extract of beef may be used instead of meat.

The addition of sugar (2 per cent.) glycerine (4 to 6 to 8 per cent.), and dyes to the culture medium is never made until just before the medium is poured into the tubes.

It is frequently necessary to give the culture medium a definite degree of alkalinity. The necessary amount of alkali is added to the medium after the latter has been rendered neutral to litmus. Thus for the cultivation of cholera vibriones, gelatine and agar-agar, after being rendered neutral to litmus, receive for each 100 cc 3 cc of a 10 per cent. solution of crystallized sodium carbonate.

PEPTONE SOLUTION

(*a*) *Preparation of the Stock Solution*

Peptone sicc.	100.0
Sodium chloride	100.0
Potassium nitrate . . .	1.0
Crystal. sodium carbonate .	2.0
Aqua dest.	1000.0

Dissolve by heating, pour into flasks (100 cc per flask), sterilize.

(*b*) *Preparation of Peptone Solution*

Tubes are filled with a dilution of 1:9 of the stock solution with water, 10 cc per tube, and sterilized.

MILK

Fresh skimmed milk which is amphoteric to litmus-paper, is poured into sterile test-tubes, and steam-sterilized for one hour on the first day, and half an hour on the two following days.

BREAD

Dry bread is pulverized, *Bread* placed in *Erlenmeyer* flasks, stirred to a thick paste, and sterilized for half an hour in the steam-sterilizer on three successive days.

THE NUTRIENT MEDIA OF CONRADI-DRIGALSKI

Seven hundred and fifty gr of meat are boiled for one hour with 900 cc of water and then filtered. To the filtrate is added water up to 900 cc, 10.0 peptone and 5.0 common salt and boiled until the peptone has dissolved; then are added 30 gr agar and, after this has dissolved, 8 to 9 cc of a 10 per cent. solution of water-free soda is added. Then it is clarified, boiled for one hour, filtered, to it is added a solution of 10 gr of nutrose in 100 cc of water thoroughly mixed, filled in flasks of 100 cc and boiled in a steam kettle for twenty minutes on two successive days.

When ready for use the agar is dissolved and cooled down to 50° C. and mixed with the following solutions:

1. Thirteen cc of a litmus solution + 1.5 gr of sugar of milk.

2. One cc of a 0.1 per cent. freshly prepared solution of crystal violet (before adding, both solutions are

boiled for fifteen minutes and cooled down to 50° C.). After mixing, the nutrient medium is poured onto plates.

ENDOS' FUCHSIN AGAR

One thousand cc neutral 3 per cent. agar nutrient medium.
Ten gr. chemically pure milk of sugar.
Five cc 0.5 per cent. alcoholic solution of fuchsin (well filtered).
Twenty-five cc of a 10 per cent. solution of natrium sulphite (freshly prepared from natrium sulphite which does not yet show any surface changes).
Ten cc of a 10 per cent. soda solution.

Five hundred gr. of beef meat is boiled for one hour with 1 litre of water, then are added 10 gr peptone, 5 gr ordinary salt and 3 gr agar and again boiled, filtered, neutralized and made alkaline by the addition of 10 cc of the soda solution.

Then are added sugar of milk and the fuchsin solution, by which the nutrient medium is stained red; then is added the solution of natrium sulphite, which gradually decolorizes the nutrient medium (the complete decolorization takes place after the agar has fully cooled). Then test-tubes are filled and sterilized in the steam kettle.

Gaethgens adds to the fuchsin 0.33 per cent. chemically pure crystalline caffein, and makes it alkaline by the addition of 15 per cent. normal sodium hydrate solution under the neutralizing point of phenolphthalein.

LOEFFLER'S MALACHITE GREEN AGAR

Five hundred gr of meat are boiled with 2 litres of water for one hour, filtered, 60 gr agar are added and boiled until dissolved, clarified, and again filtered. If the agar does not dissolve well, 14 cc of normal HCl are added, which are neutralized with 14 cc normal potassium hydrate after the agar has dissolved. Then natrium carbonate is added until it reacts neutral to litmus. After neutralization 25 cc normal soda is added and the weak alkaline solution is boiled up. To this boiling hot mass are added 200 cc of a 10 per cent. watery solution of nutrose. After it has been boiled up again the hot solution is filled in flasks of 100 cc and sterilized for twenty minutes on two successive days. To 100 cc of the liquefied, clear bouillon nutrose agar which has been cooled down to 50° C. are added before use 1.5 cc of a 0.2 per cent. solution of malachite green crystals, chemically pure. The green agar is poured into Petri dishes which are left open until it cools and solidifies. If cultures are intended from fæces, *Loeffler* recommends the addition of 3 per cent. beef gall. But then 1.9 cc malachite green must be added instead of 0.5 cc.

GREEN SOLUTION I

Nutrose, 1.0; peptone, 2.0; grape-sugar, 1.0; sugar of milk, 5.0; aqua dest., 100; 0.2 per cent. solution malachite green crystals, chemically pure, 1.0; normal potassium hydrate, 1.5.

GREEN SOLUTION II

Nutrose, 1.0; peptone, 2.0; sugar of milk, 5.0; normal potassium hydrate, 1.5; "malachite green 120," 2 per cent. 3 cc; aqua dest., 100.0.

The solutions were prepared from 10 to 20 per cent. stock solutions of the several ingredients, so that at first the peptone, the grape- and milk-sugar were mixed together, then were added the potassium hydrate and after this the nutrose, and finally the green stain.

V. Lingelsheim's Litmus Ascites-Sugar Agar

Ten cc of a 10 per cent. solution of the sugar to be examined in *Kubel-Tiemann's* litmus solution are put into test-tubes and heated for two minutes in a water-bath at 100° C. After cooling are added to each 10 cc, 0.5 cc normal soda solution. Of this 1.5 cc are added to each 13.5 cc of liquid ascites agar (1 part ascites, 3 parts agar). The nutrient medium is poured into *Petri* dishes.

NEUTRAL-RED AGAR

For every 100 cc of agar, 0.3 gramme of grape-sugar and 1 cc of a saturated watery solution of neutral-red are added, before the agar is poured into tubes.

PETRUSCHKY'S LITMUS-WHEY

Warm milk is diluted with an equal quantity of water, and treated with sufficient dilute HCl to precipitate all the casein. A measured quantity is at first tested to ascertain how much HCl is necessary to just coagulate the milk, and the amount necessary to coagulate the entire quantity is calculated from it. The casein is removed by filtration; the filtrate neutralized with soda solution, boiled for one to two hours in the steam-sterilizer, and filtered. The reaction is again tested, it is rendered exactly neutral, and again boiled. It is then treated with a sterile tincture of litmus until it is violet in color, poured into tubes, and sterilized.

BARSIEKOW'S CULTURE MEDIUM

Nutrose	1.0
Milk-sugar	1.0
Sodium chloride	0.5
Aqua dest.	100.0
Adde litmus solution . . .	5.0

Or, instead of milk-sugar 1.0, grape-sugar 1.0, or grape-sugar and milk-sugar āā 1.0.

The sugar-nutrose sodium chloride solution is boiled twenty minutes in the steam-sterilizer, filtered, and after the addition of the litmus solution poured into tubes and sterilized twenty minutes.

Blood-Agar

According to *Pfeiffer* blood-agar is prepared with human or pigeon blood. The former is obtained by pricking the ball of the finger or lobe of the ear, after disinfecting the skin with alcohol and ether, the latter from the large vein of the pigeon's wing, which is opened after the feathers have been removed and the skin cleansed. The drop of blood is taken with the platinum loop, as it issues, and smeared upon the surface of congealed agar. The culture medium is placed for twenty-four hours in the incubator at 37° C., in order to test its sterility.

Czaplewski's Blood-Agar

Pigeon-blood, which has been obtained under aseptic conditions, is mixed in an *Erlenmeyer* flask with liquefied agar, which has been cooled to 50° C., thoroughly shaken, and liquefied agar added until the medium appears but slightly red. After any clots which may have formed have been removed with the platinum needle, the medium is at once poured into small *Petri* dishes, or tubes, in which it is allowed to solidify obliquely. Before using, the plates are dried, inverted and open, for a short time in the thermostat at 50° C.

Hesse's Agar

Five grammes of sodium chloride, 10 grammes of agar-agar, 30 cc of glycerine, and 5 cc of normal sodium carbonate solution are covered with 1,000 cc of water and boiled for two hours in the steam-sterilizer. Five grammes of *Heyden's* food, mixed with water, are added, and the mixture boiled for a quarter of an hour in a water-bath, filtered, poured into tubes, and sterilized in the usual manner.

Blood Serum

When possible the blood is obtained under aseptic precautions by allowing it to run through a sterile rubber tube and a cannula, which is introduced into the carotid of an animal, into sterile glass receptacles, which can be tightly closed. The receptacles containing the blood are placed at once in an ice-chest (temperature 7° to 8° C.). After the blood has coagulated, the clot is loosened from the sides of the glass with a sterile glass rod. After one to three days the serum, which has separated, is removed with a sterile pipette and placed in *Petri* dishes (about 20 cc per dish) and tubes (about 5 cc per tube). Serum which is not to be used at once may be placed in sterile *Erlenmeyer* flasks, and after the addition of about 2 per cent. chloroform, kept in an ice-chest. The *Petri* dishes and the tubes are placed for two hours in a thermostat at 60° to 65° C., in order to solidify the serum. The solidified serum is transparent and amber-yellow in color. The medium is tested for its sterility by placing in the incubator at 37° C.

When it is impossible to obtain the blood in an aseptic manner, it is obtained when an animal is slaughtered, from a stab-wound made through a clean, or at least moistened, area, and collected in sterile glass receptacles. The first blood which issues is allowed to escape, since the hair, etc., from the area surrounding the wound is washed by it. The further treatment of the blood is the same as that described above. The serum must, however, either be sterilized in its fluid state, by placing it in a thermostat at 55° to 58° C. for four to five hours on eight successive days, or must be sterilized, after it has solidified, for two hours on three successive days at 65° to 68° C. In order to prevent too great drying of the serum, a vessel of water is placed in the thermostat with it. The majority of plates and tubes so prepared will be found sterile when tested in the incubator.

Loeffler's Serum

Three parts of ox or sheep serum are mixed with 1 per cent. slightly alkaline grape-sugar bouillon. The mixture is coagulated and sterilized in a steam-sterilizer, which, in order to avoid the formation of bubbles in the medium, is so gradually heated that the serum has solidified before the water begins to boil. *Neisser* has suggested a special serum oven. The medium so obtained is not always sterile, and must therefore be tested before use by placing in the incubator at 37° C. Serum tubes are sterilized in the steam-sterilizer for a quarter of an hour on the two following days. Plates must be kept inverted, since considerable condensation-water is expressed.

Blood-Serum Agar

Fluid blood-serum, which has either been obtained in an aseptic manner, or sterilized by fractional sterilization at 55° C. (eight days, four hours a day), is heated to 40° to 50° C., and mixed in the ratio 1: 2, with 2 to 3 per cent. agar or glycerine-agar, which has been melted and cooled to 50° C. The mixture is poured into *Petri* dishes, or into obliquely placed tubes, and allowed to solidify.

Wertheim's Human Blood-Serum Agar

Human blood is obtained by venesection or from the placenta. After the cord has been tied or cut, the maternal end is disinfected with corrosive sublimate, washed with distilled water, and again cut above the knot. The blood which issues is collected in sterile flasks, and the serum kept in its liquid state, with the addition of chloroform, and used for the preparation of culture media, is treated in the above manner with agar, in the ratio of 1:2 or 1:3, shortly before it is to be used. The mixture is allowed to solidify in slanting tubes.

Ascites-Agar

The serous fluid obtained by puncture is treated with 2 to 3 per cent. chloroform, kept in a cool and dark place, and frequently shaken. When the fluid has become absolutely clear, it is withdrawn with a sterile pipette, and placed in tubes. Before using, the chloroform is driven off by heating to 35° C. (in a water-bath or incubator). The fluid is mixed with the agar in the same manner as in preparing blood-serum agar.

Kiefer's Ascites-Agar

Ascites fluid is heated to 50° C. shortly before it is to be used, mixed with an equal quantity of neutral liquefied glycerine-agar, which has been colored to 50° C., and contains 3.5 per cent. agar, 5 per cent. peptone, 0.5 per cent. sodium chloride, and 2 per cent. glycerine, and poured into *Petri* dishes. If the ascites fluid is strongly alkaline, the agar is either not previously neutralized, or is sufficiently acidified to give the mixture a slight alkaline reaction.

BEER-WORT CULTURE MEDIA

After sterilization in a steam-sterilizer, the beer-wort is set aside for some time, the clear fluid decanted into tubes and again sterilized. By the addition of 10 per cent. gelatine, or 2 per cent. agar, beer-wort gelatine or agar are obtained. The culture media are not neutralized.

All culture media must be tested as to their sterility before using. To this end they are placed for twenty-four hours in the incubator. Culture media whose base is gelatine must only be exposed to temperatures between 20° and 25° C. Agar-agar mixtures, blood-serum, potato, and fluid media may be kept at higher temperatures.

The Cultural Methods Most Frequently Employed

Aerobic Cultures

PLATE CULTURES

(*a*) GELATINE PLATE CULTURES.—Three tubes of gelatine are liquefied in a water-bath at 30° to 35° C. One of them is removed and held by the tip between the thumb and forefinger of the left hand (with the volar side up) as obliquely as possible; the cotton plug removed and held between the third and fourth fingers of the left hand, so that the portion which belongs within the tube does not touch the skin. The material to be inoculated is then introduced into the gelatine by means of the platinum loop, which is held like a pen, and has been sterilized in the flame and again cooled. Fluid material is mixed directly with the gelatine; solid material is first smeared upon the side of the tube and gradually mixed with the gelatine. After the cotton plug has been singed, it is replaced, and, by carefully tipping and turning the tube, the material is distributed as evenly as possible throughout the fluid medium, without allowing the latter to touch the cotton plug. The tube is again held in the above-described manner, with a second tube parallel to it. Both are opened, and one or more loops, depending upon the number of bacteria contained in the material to be examined, are transferred from the first to the second tube; both tubes are again closed, and the first tube is replaced in the water-bath. After the contents of the second tube have been carefully mixed, several loops are transferred from it to a third tube. After the mouths of the tubes have been burned and allowed to cool, the inoculated gelatine is then poured into sterile *Petri* dishes, whose covers are raised at one side only just high enough to allow of it. The gelatine is again mixed by carefully rocking the plates. The plates are marked O (original plate) 1 and 2 (first and second dilution), and with the date of inoculation, allowed to solidify upon ice, and placed in a thermostat at 22° C.

(*b*) AGAR PLATE CULTURES.—Agar may be inoculated in the same manner as gelatine, but must first be melted in boiling water, and again cooled to 50° C.

SURFACE CULTURES

The material to be examined is placed upon the medium contained in *Petri* dishes, and spread evenly in all directions over its surface by means of a platinum needle, which has been bent so that it is parallel to the surface, or a right-angled glass spatula. The glass spatula may be sterilized by burning alcohol on it. If the material to be inoculated contains a large number of bacteria, it is necessary, in order to obtain isolated colonies, either to first mix it with a sterile fluid (physiological salt solution or bouillon), and use a loop of the mixture for inoculating, or to smear several (three to four) plates, one after another, with the same loop, without touching it again to the material. The plates are placed, inverted and open, in the incubator some time before they are inoculated, in order to allow the condensation-water to evaporate. *Petri* dishes are also inverted before inoculating; the dish is removed from the cover, and the culture medium smeared with its surface down.

Inoculation of Tubes containing Slanting Media (Agar, Blood-Serum, etc.)

A small quantity of the material to be examined is smeared over the surface of the medium with a platinum wire, while the tubes are held horizontally. To obtain isolated colonies several tubes are smeared, one after the other, with the same loop.

Stab Cultures

Tubes containing culture medium, which solidified while they were in a vertical position, are held horizontally, and the medium stabbed with a platinum needle carrying the bacteria to be cultivated.

"Schuettel" Cultures

The medium is melted in a water-bath (agar must be cooled to 50° C.), a loop from a pure culture is introduced, the tube thoroughly shaken, and the medium allowed to solidify while the tube is in a vertical position.

Inoculation of Fluid Culture Media

This is accomplished in the same manner as that of melted gelatine.

Anaerobic Cultures

It is advisable to add reducing substances such as 1 to 2 per cent. grape-sugar, 0.3 to 0.5 per cent. sodium formate, or 0.1 per cent. sodium indigo-sulphate, to culture media which are to be used for cultivating anaerobic bacteria.

Various methods are used for cultivating bacteria in the absence of air.

(*a*) *Mechanical Exclusion of Air*

1. A thin sheet of mica, which must be at least large enough to cover one-third of the surface of the medium, is placed in the centre of the inoculated agar, or gelatine, plates, just as the medium commences to solidify.

2. *Inoculation of a Deep Layer.*—Well-filled agar, or gelatine, tubes are boiled half an hour in a water-bath, in order to expel the air, quickly cooled, and inoculated with the material to be examined. After the medium has solidified (on ice), it is covered with a layer of agar or gelatine.

For examination, the tubes are broken and the medium sliced with a sterile knife.

To obtain pure cultures, stab cultures in well-filled agar or gelatine tubes, which have been boiled and quickly cooled on ice, are made, and the medium likewise covered with a layer of sterile medium after the inoculation. The inoculation must be made with a long needle which reaches deep down into the medium.

Material is taken for examination from stab cultures from the depths of the stab canal, which is entered from above, without breaking the tubes.

(*b*) *Removal of the Air by Means of an Exhaust Pump*

Large tubes are drawn out into a thin tube at a point in their upper third, filled with the culture medium (the constricted point must remain dry), sterilized, and inoculated in the usual manner. The cotton plug is then pressed deep down into the neck of the tube, and the tube closed with a tightly fitting rubber stopper, through which a right-angled glass tube passes. The tube is connected with an exhaust pump. During the exhaustion of the air, the culture medium is placed in a water-bath at 30° to 35° C. for gelatine, at 42° C. for agar. As soon as the air is exhausted (after about fifteen minutes), the constricted part of the tube is closed by melting.

(*c*) *Removal of the Oxygen from the Air by Chemical Means*

The inoculated tube is closed with a cotton plug, and placed on a wire shelf in a second wide tube, which contains an alkaline solution of pyrogallol. (For every 100 cc of air space 1 gramme of pyrogallic acid, and shortly before the vessel is closed, 10 cc of a solution of one part liquor potassæ in ten parts of water. The outer vessel is hermetically closed with a rubber stopper and sealed with liquefied paraffin. The absorption of the oxygen requires about twenty-four hours, during which time the cultures are kept at ordinary temperature.

This procedure may also be used for plate cultures by using a jar with a ground top.

(*d*) *Replacement of the Air by Hydrogen*

The tube or flask containing the inoculated medium is closed with a rubber stopper, through which pass two right-angled glass tubes, one of which extends into the culture medium, while the other extends but a trifle below the stopper. The outer arms of the tubes are drawn out to capillary tubes. The longer tube is joined to a *Kipp's* hydrogen generator, and hydrogen run through it until the oxygen is driven off. The capillary tubes are then closed by melting.

For plate cultures, either *Kitasato's* plates or *Botkin's* apparatus is used. The latter consists of a deep glass dish, containing a glass bell, which rests on a metal cross. Two U-shaped tubes pass at opposite sides of the bell between its rim and the bottom of the dish. They serve for the introduction of hydrogen and the exit of the air, and after the latter has been completely driven off, they are closed by melting. The dish is filled with liquefied paraffin, in order to exclude air. The inoculated plates are placed open upon a wire shelf within the bell. A dish, containing an alkaline solution of pyrogallol, is also placed within the bell.

V. Determination of the Biological Characteristics of Bacteria

Detection of Peptonizing Ferments.—This is accomplished by means of gelatine stab cultures; gelatine is liquefied by the action of peptonizing ferments.

Determination of Fermentative Power.—This is accomplished by means of stab cultures in sugar-agar or by the inoculation of sugar-bouillon, which is poured into fermentation flasks. The fermentation flasks, which have been sterilized in a dry sterilizer, are filled with sterile, 2 per cent. grape-sugar bouillon, and before using are again sterilized for half an hour in the steam-sterilizer.

Detection of Acid or Alkali Formation.—This is accomplished by the addition of an indicator—for example, tincture of litmus—to neutral culture media. *Petruschky's* litmus whey and *Barsiekow's* culture media are largely used for this purpose.

Detection of Indol Formation.—Cf. p. 112.

Detection of Hydrogen Sulphide Formation.—A piece of moistened lead paper is placed between the cotton plug and the culture tube, so that it protrudes into the latter. If hydrogen sulphide is formed, the paper turns black.

Determination of Reducing Power.—A dye which is decolorized by reduction (methylene blue, litmus, neutral red) is added to the sterile culture media.

To Determine whether Bacteria are Aerobic or Anaerobic Inoculation of a Deep Layer is Used.—(Cf. p. 359.)

Determination of Toxin Formation.—Detection of extracellular toxins: The culture fluid, which contains the toxins in solution, is filtered free from bacteria,[1] and injected into test-animals in measured doses. Detection of intracellular toxins: The bacteria are cultivated upon solid media and killed, removed with a normal loop (capacity of 2 milligrammes) without admixture of media, and mixed with a measured quantity of sterile fluid. Definite quantities of this mixture, or a dilution of it, are then used for animal inoculation. In this manner an entire loop, or $\frac{1}{100}$, $\frac{1}{1000}$, etc., of a loop, may be injected. The bacteria are killed by two hours' stay in the thermostat at 60° C., or by chloroform vapor. The bottom of the cotton plug of the culture tubes is moistened with chloroform, replaced, the tubes closed with double rubber caps, and kept for several hours in the incubator at 37° C. Before the test is made, the fact that the bacteria have been killed must be established by transplantation upon other culture media, which must remain sterile.

---

[1] Bacteria-proof filters (*Chamberland* filter, filters of infusorial earth, etc.) are used, through which the fluid is drawn by means of a suction-pump.

VI. Methods of Animal Inoculation

1. *Cutaneous Inoculation.*

The skin is shaved, disinfected, freed from the disinfectant, and may or may not be slightly scarified. The material to be examined is then rubbed into it with a sterile instrument.

2. *Subcutaneous Inoculation.*

(*a*) *Inoculation by Injection.*—Fluid material is injected directly with a hypodermic syringe, solid material after admixture with sterile physiological salt solution or bouillon.

(*b*) *Inoculation in a Pocket under the Skin.*—The skin is disinfected, raised with a thumb-forceps, the fold thus produced snipped with scissors, and the material to be examined introduced through the nick. If necessary, the wound is closed with collodion. Mice and rats are usually inoculated just above the base of the tail, guinea-pigs on the side of the abdomen or chest, rabbits on the inner side of the ear.

3. *Inoculation in the Large Cavities of the Body.*

A slight nick is made in the skin, and the material injected with a syringe having a dull needle. The needle is introduced into the abdominal cavity in the median line; into the pleural cavity at the upper edge of a rib.

4. *Inoculation into the Bloodvessels.*

In rabbits the injection is made into one of the large veins at the border of the ear; in larger animals into the jugular vein.

5. ***Inoculation into the Anterior Chamber of the Eye.***—(Cf. p. 20.)

6. ***Inoculation by Means of Food.***

The material to be examined is mixed with the food.

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INDEX

Acetic acid in stomach, 62
Acetone in urine, 163
    test of, 204
Actinomycosis, 28, 31
Adventitious constituents of the urine, 175
Agar medium, 346
Agglutination, 272
Albumin, detection of, 142, 180
    in urine, 141
    precipitate in cold, 150
Albuminosis, 148
Almen's test, 172
Aloin test for blood, 67
Alveolar epithelial cells, 33
Ammonia test in urine, 203
Amœba in fæces, 97
Amylum, 96
Anaerobic cultures, 359
Anchylostoma duodenale, 103
Angina Vincenti (Plautii), 11
Animal test for tubercle bacilli in urine, 242
Animal tests, methods of, 363
Anthrax bacillus, 128, 301
Anthrax carbuncle, 301
Antigen, syphilitic, 280
Antipyrin in urine, 178
Apparatus for collecting material, 1
Arsenic in urine, 176
Ascaris lumbricoides, 102
Ascites agar, 355
Babes-Ernst's bodies, 3
Bacillus fusiformis, 11
    pyocyaneus, 52
Bacteriological examination in diseases of the skin, 299
    of conjunctival secretions, 19
    of fæces, 104
    of fluids by puncture, 291
    in nasal secretions, 17
    in secretions of the mouth, 1
    in sputum, 37
    in urine, 237
Bacteriolysine, 275
Bacterium coli in urine, 239
Balantidium coli, 98
Barsiekow's medium, 108, 351
Baumgarten's method of staining lepra bacilli, 332
Benzidin test, 67, 82, 172
Biliary acids, 83
Biliary concretions, 90
Biliary pigments in fæces, 82
    pigments in gallstones, 90
    pigments in stomach, 66
    pigments in urine, 168
Biological properties of bacteria, test of, 361
Biuretic reaction, 149
Boiling test for albumin in urine, 145

Blood, examination of, 250
  in contents of stomach, 66
  in fæces, 82
  in urine, 170
  pigment, 170
  serum agar, 354
  serum as medium, 353
  specimens, staining of, 256, 338
Blood-agar, 352
Blood-cells, morphology of, 259
Blood-corpuscles, counting of, 253
Boas "faden" (threads), 73
Borax methylene blue, 329
Bothriocephalus latus, 100
Bouillon as medium, 345
Brandberg's method of testing albumin, 180
Bread as medium, 348
Butyric acid in stomach, 62

Capsules of anthrax bacilli, staining of, 335
Carbohydrates, in fæces, indirect estimation of, 86
  in urine, 151
Carbol fuchsin, 329
Casts in urine, 230
Celloidin for imbedding, 340
Cercomonas intestinalis, 98
Cerebro spinal fluid, 289
Charcot-Leyden crystals, 30, 36
Chloride in urine, 199
Cholera vibriones, 120
  test of, in fæces, 123
Cholesterine in gallstones, 91
Chromogen of the urine, 166
Chrysophanic acid, 177
Coatings of mouth and pharynx, 1
Collection of material, 1
Congo-paper test, 59
Conjunctival secretion, 19
Conradi-Drigalski medium, 106, 348
Copaiba balsam in urine, 179
Creatin, test for, in urine, 132
Culture media, 344
Culture method, 356
Curschmann's spirals, 27, 29
Cut sections, 339
Cylindroids in urine, 232
Czaplewski's method of counting tubercle bacilli in sputum, 40
  of staining same, 331

Dermato-mycosis, 305
Dextrose in urine, 151
Diacetic acid, 163
Diazo reaction, 174
Dimethylamidoazobenzol, test with, 60
Diphtheria bacilli, 2-8
  in conjunctival secretions, 19
  in nasal secretions, 17
  staining of, 332
Diplobacillus of *Friedlaender*, 17, 52
  of *Morax* and *Axenfeld*, 21
Diplococcus flavus, 16
Dittrich's plugs, 27, 30
Donne's test for pus, 135
Ducrey's bacillus, 304, 343
Dysentery bacillus, 117

Echinococcus cysts, 288
  hooks, 31
  in urine, 235
Eggs of intestinal parasites, 99–103
Elastic fibres in sputum, 35
Endos fuchsin agar, 104, 349
Enteroliths, 92
Epithelium in fæces, 97
  in urine, 224
Erythrasma, 316
Essbach's method of estimating albumin, 181
Exudates, 286

Fæces, examination of, 74
  dry matter, 84
Fat, in fæces, 81, 85, 96
  in urine, 134
Fatty acids, volatile, in stomach,
Favus, 309
Fecal balls, 83
Fecal concretions, 92
Fecal sieve, 76
Fehling's test, 156
Fermentation test according to *Roberts*, 183
  to *Lohnstein*, 184
Fibrin in urine, 151, 229
Ficker's diagnosticum, 275
Flagella, staining of, 336
Formic acid in gastric contents, 62
Freeing the urine of albumin, 149
Freezing-point of urine, how to determine, 137
  of blood, 251
Fruit sugar, 160
Fungi, staining of, 337
Gabbet-Fraenkel's method of staining tubercle bacilli, 331
Gaffky's scale, 41
Gastric contents, examination of, 56, 68
Gelatine as medium, 346
Gerhardt's test, 164
Giemsa stain, 258, 339
Glucose in urine, 151
Glycuronic acid, 162
Gmelin's test, 83, 168
Gonococci, cultivation of, 247
  in conjunctival secretions, 20
  in urine, 246
Gonorrhœal threads, 234
Gram's staining method, 330, 342
Grape-sugar in urine, 151
Gravimetric analysis, 182
Green solution, *Loeffler's*, 112, 349
Guaiacum test after *Weber*, 66, 82
Guensburg's reaction of HCl, 59
Gutceit's detection of arsenic, 176

Hæmato-porphyrin, 173
Hæmoglobin in urine, 170
  test in blood of, 251
Hæmolytic system, 282
Hæmosyderine, 34
Hair, examination of, 306
Hanging drop, 327
Heart-failure cells, 34
Heller's test of albumin, 143
  of blood, 171
Hesse's medium, 44, 352

Hopkins' method of estimating uric acid, 193
Huppert's test for biliary pigment, 83, 169
Hyaline casts in urine, 231
Hydrobilirubin in fæces, 83
Hydrochloric acid, estimation of, 69
  free, 58
  total, 70
Hydrogen-sulphide, 68
Hydronephrosis, 288

Indican in urine, 166
Indol reaction, 112
Indoxyl sulphuric acid, 165
Influenza bacillus, 51
Infusoria in fæces, 98
Inoscopy, 293
Intestinal gravel, 93
  parasites, 97
Iodine test in urine, 177

Jacobi's method of determining pepsin, 63

Kernels in sputum, 26
Kiefer's ascites agar, 355
Kjeldahl's method of estimating nitrogen in fæces, 85
  in urine, 187
Koch-Week's bacillus, 20
Kowarsky's copper plate, 257
  method of determining uric acid, 193
  pheryl-hydrozin test, 158
Kuehne-Weigert's method of cut staining, 338, 342

Lactic acid, estimation of, 72
  in the urine, 160
Lactic acid, test of, 60
Lactose in urine, 160
Lecithin granules, 235
Legal's test of acetone, 163
Leischmann's stain, 259
Lepra bacillus, 18
Levaditi's method, 322
Levulose in urine, 160
Lieben's test for acetone, 164
Liebermann-Allihn's estimation of starch in fæces, 87
Litmus-ascites-sugar agar, 351
  litmus-whey, 108
Liver extract, 280
Loeffler's methylene blue, 3
  serum, 4, 354
Lohnstein's saccharometer, 184
Ludwig-Salkowski's method of estimating uric acid, 197
Malachite-green agar, 107, 349
Malaria parasites, 263
Mallei bacilli, 300
Manson's method of staining, 264
May-Gruenwald stain, 258
Meat-poisoning bacillus, 117
Melanin in urine, 174
Meningococci, 13, 296
Mercury in urine, 175
Metahæmoglobin, 170
Methyl violet as reagent for HCl, 60
Mett's method of determining pepsin, 63
Micrococcus catarrhalis, 15, 51
  cinereus, 16
  tetragenus, 50
  ureæ, 136

Microscopical examination of blood, 255
  of fæces, 94
  of gastric contents, 72
  of gonococci, 247
  of urine, 209
Microsporia, 312
Microsporon furfur, 315
Milk as a medium, 348
Milk-sugar, 160
Minz's estimation of hydrochloric acid, 69
Mouth, secretions of the, 1
  spirochetæ of the, 12
Mucin in fæces, 80
Mucus in fæces, 78, 96
Muscle fibres in fæces, 95

Nasal secretions, 17
Neisser's stain, 3, 333
Neutral red agar, 109, 351
Nitrogen, total, estimation of, 85
Nutrose litmus bouillon, 7
Nylander's test, 152

Oidium albicans, 10
Onychomycosis, 314
Oppler-Boas threads, 73
Ovarial cysts, 314
Oxalate calcium in urine, 214
Oxalic acid, determination of, 202
β-Oxybutyric acid, 163
Oxyhæmoglobin, 170
Oxyuris vermicularis, 101

Pancreatic cysts, 289
Pancreatic stones, 93
Pappenheim's method of staining gonococci, 334
  tubercle bacilli, 332
Paraffin embedding, 339
Paratyphoid bacillus, 115, 273
Pavy-Sahli method of estimating sugar, 185
Pentaglucose, 161
Pentose, 161
Pepsin, 62
Pepsinogen, 62
Peptone solution, 347
Peptones, 148
Petruschky's litmus-whey, 351
Pfeiffer's test, 275
Pharynx, 1
Phenacetin in urine, 178
Phenol in urine, 179
Phenyl-hydrazin test, 158
Phosphate in sediment of urine, 216
  estimation of, 199
Pigment of urine, 165
Pityriasis versicolor, 315
Plague bacillus, 53, 128
Plate cultures, 356
Plaut-Vincent's angina, 11
Pneumo-bacillus, 52
Pneumococci, 48
Polar granules, 3
Polarization, 183
Potassium bromide in urine, 177
Potassium iodide in urine, 177
Potatoes as medium, 344
Prostate, secretions of, 235, 249
Proteus vulgaris, 245
  Hauseri, 136
Pseudo-diphtheria bacillus, 5
Pseudo-mucin, 287

Pus corpuscles in fæces, 97
    in urine, 227

Reaction of fæces, 80
    of gastric contents, 58
    of urine, 135
Red blood-corpuscles, counting of, 253
    in fæces, 97
    in urine, 229
    morphology of, 259
Relapsing fever, spirilla of, 268
Renin, 65
Reninogen, 65
Roberts' and *Stolnikoff's* method, 180
Roberts' fermentation test, 183
Rosenbach modification, 169
Rosin's test, 169
Roux's solution, 3, 333

Saccharometer (*Lohnstein's*), 185
Salicylic acid in urine, 177
Sarcinæ, 72
Schlesinger's method, 167
"Schuettel" cultures, 358
Sedimentation, 42
Sedimentum lateritium, 134
Serum diagnosis, 272
Skin diseases, 299
Smegma bacillus, 241
Soor fungus, 10
Spectroscopical tests, 67, 82, 172
Spermatozoa, 235
Spiegler's test of albumin, 146
Spiral cells, 72
Spirocheta buccalis, 12
Spirocheta pallida, 317–326
    various methods of staining of, 320–324
Spores, staining of, 334
Sputum, examination of, 22
Stab cultures, 358
Staining methods, 329
    solutions, 329
Staphylococci in fæces, 127
    sputum, 50
    urine, 240
Starch in fæces, estimation of, 87
Stomach, *see* Gastric Contents
Stomatitis ulcerosa, 13
Stones, composed of drugs, 93
    light, 93
Streptococci in fæces, 127
    in sputum, 49
    in urine, 240
Stukowenkoff's detection of mercury, 175
Sugar, estimation of, 182
Sulphates in urine, 201
Sulphosalicylic acid test, 145
Sykosis, 312

Tænia, cucumerina, 101
    flavopunctata, 101
    nana, 101
    saginata, 99
    solium, 99
Test-breakfast of *Ewald*, 56
Tetanus bacillus, 302
Toepfer's estimation of free hydrochloric acid, 69
Transudates, 286
Trichocephalus dispar, 102
Trichomonas intestinalis, 98
Trichophytosis, 311
Trommer's test, 154

Tubercle bacilli in fæces, 126
    in sputum, 40, 46
    in urine, 240
    in secretions of conjunctiva, 19
    in secretions of nose, 17
    staining of, 330, 343
Typhoid bacilli in blood, 269
    in fæces, 104, 113
    in urine, 244

Urates, 212
Urea, 131, 193
Urethral secretions, 245
Uric acid, 131
    in sediment, 211
Urinary calculi, 206
Urinary concretions, 206
Urine, casts of, 230
    collection of, 129
    filaments of, 234
Urine, general properties of, 132, 141
    identification of, 130
    sediment of, microscopic examination of, 209
    specific gravity of, 136
Urobilin, 167
Urobilinogen, 167
Urotropin in urine, 179

Wassermann's reaction, 278
Weber's test for blood, 66, 82
Widal's reaction, 274

Xanthin stones, 206, 208
Xerosis bacilli, 8

Ziehl-Neelsen's staining of tubercle bacilli, 330
Ziehl's solution, 2, 11, 329

PLATE I

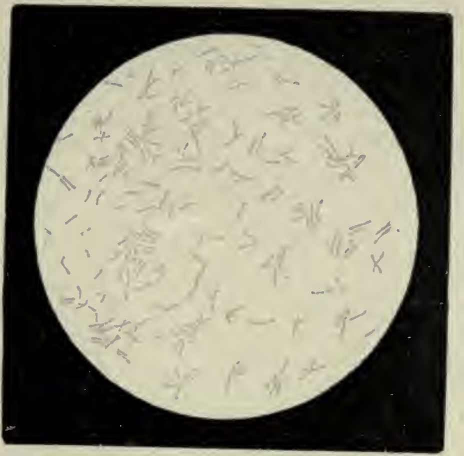

FIG. A.—Diphtheria Bacilli from a Serum Plate. Stained according to *Roux*. Magnification, 1:1,000. (After *Czaplewski.*) See page 3.

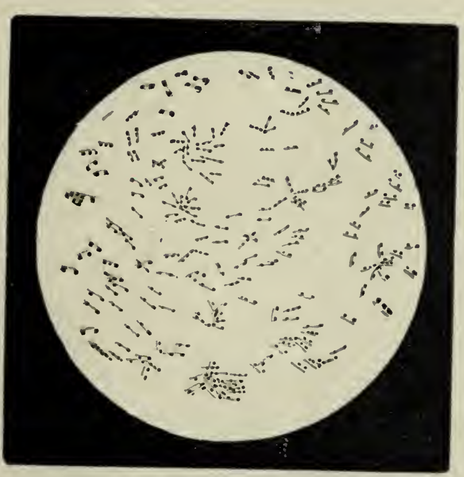

FIG. B.—Diphtheria Bacilli from a Serum Plate. Stained according to *Neisser*. Magnification, 1:1,000. (After *Czaplewski.*) See page 3.

PLATE II

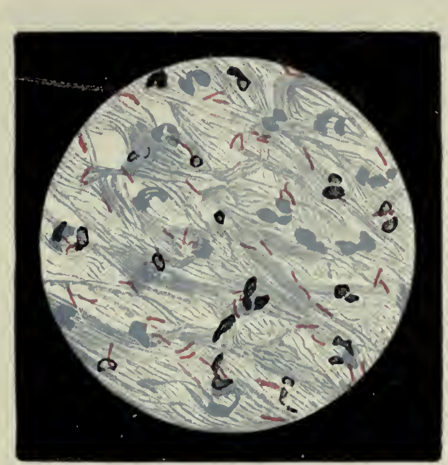

FIG. C.—Smear from Sputum containing numerous Tubercle Bacilli. Stained according to *Ziehl-Neelsen.* After *Czaplewski.*) See page 40.

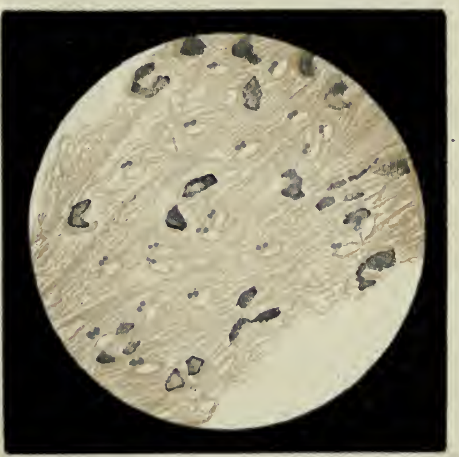

FIG. D.—Smear from Pneumonic Sputum. Stained according to *Gram.* (After *Czaplewski.*) See page 48.

PLATE III

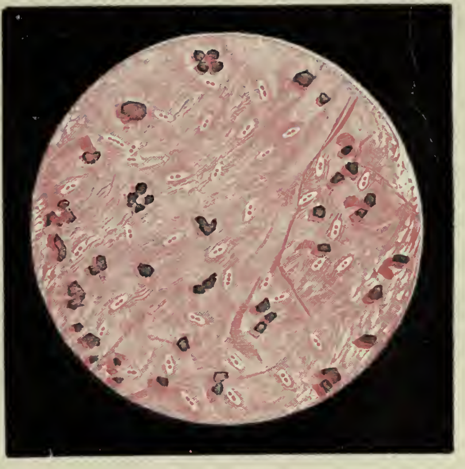

FIG. E.—Smear from Pneumonic Sputum. Stained with Carbol-fuchsin. (After *Czaplewski.*) See page 48.

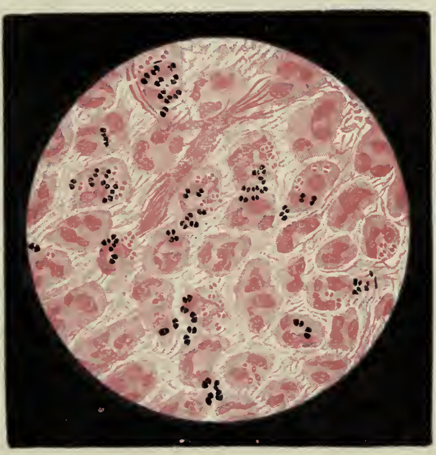

FIG. F.—Smear from Bronchial Sputum in a Case of Catarrhal Bronchitis. (Specimen from *Professor Kolle.*) Stained with dilute Carbol-fuchsin. Magnification, 1:1,000. (After *Czaplewski.*) See page 51.

PLATE IV

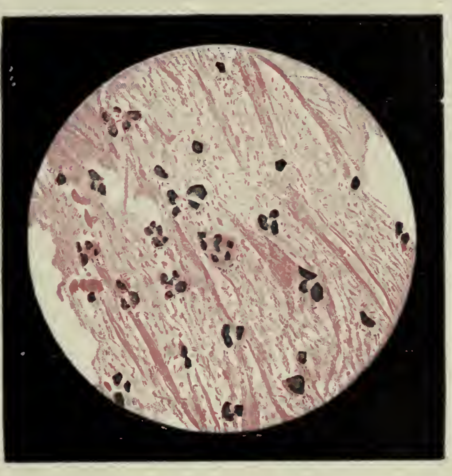

FIG. G.—Smear from Pulmonary Sputum containing Influenza Bacilli. Stained with fuchsin. (Specimen from *Professor Kolle,* drawn by Landsberg, Berlin.) See page 51.

PLATE V

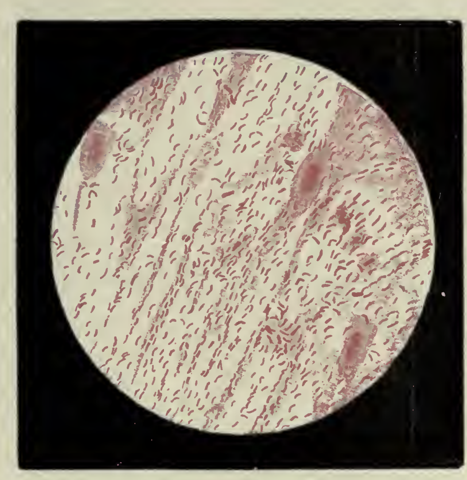

FIG. H.—Smear from a Cholera Dejection. A Mucus Fleck containing an almost Pure Culture of Comma Bacilli. The Shoal arrangement. Stained with Dilute Carbol-fuchsin. Magnification, 1:500. (After *Kolle.*) See page 120.

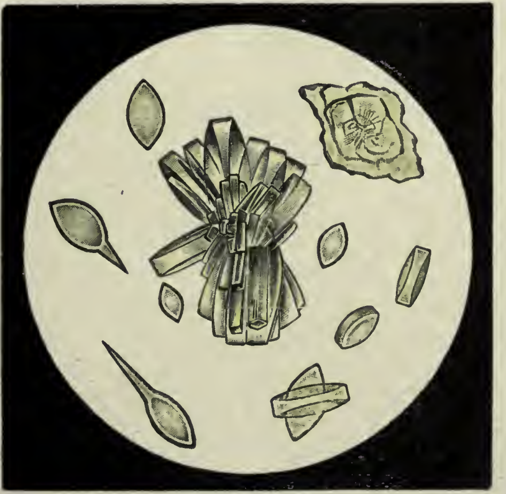

FIG. I.—Uric Acid. See page 211.

PLATE VI

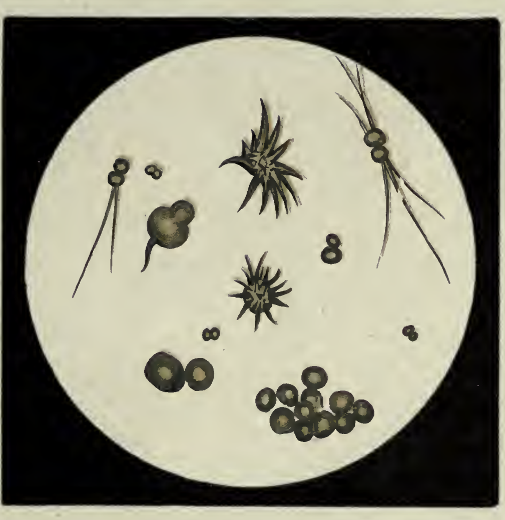

FIG. J.—Ammonium Urate. See page 213.

PLATE VII

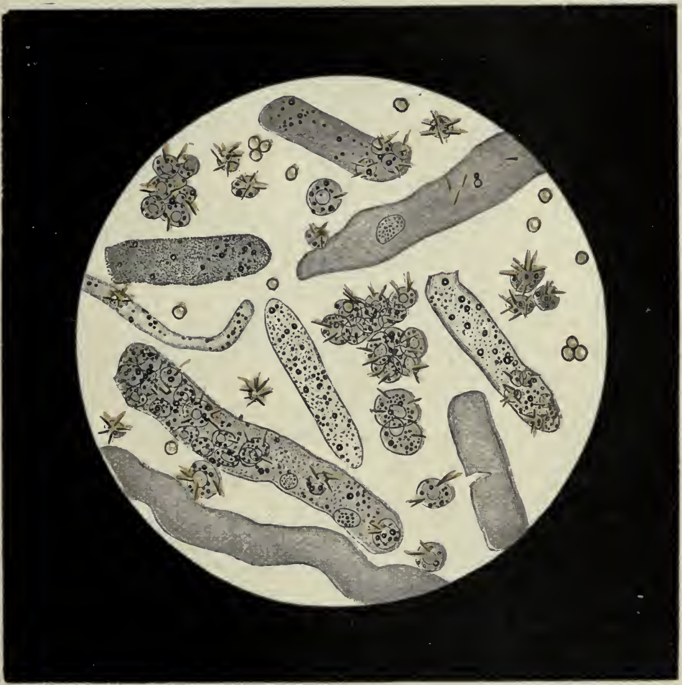

FIG. K.—Nephritis in Pregnancy. Eclampsia. Hematoidin Crystals. (After *Blumenthal.*) See page 222.

PLATE VIII

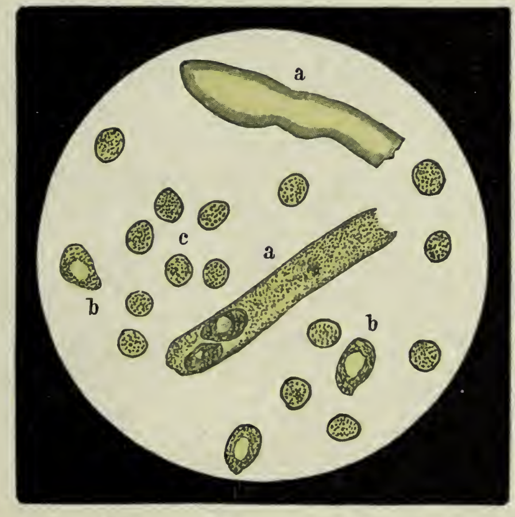

FIG. L.—*a*, Casts from icteric urine; *b*, Renal epithelial cells from icteric urine; *c*, Leucocytes from icteric urine. See page 230.

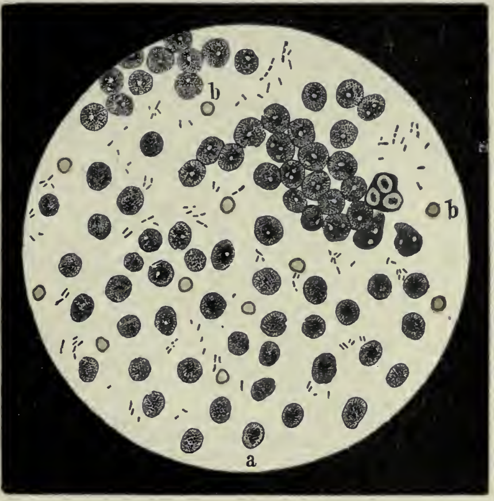

FIG. M.—*a*, Leucocytes (pus-corpuscles) ; *b*, Red blood-corpuscles. See page 227.

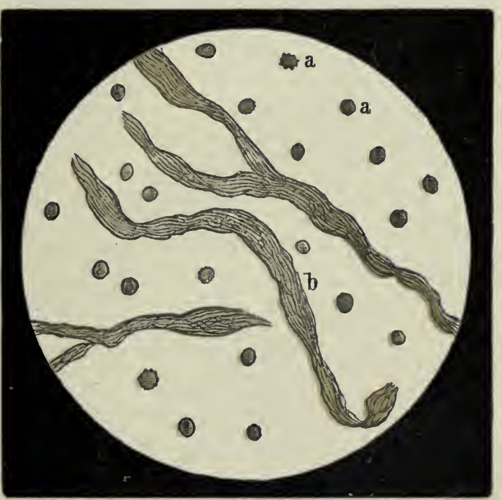

FIG. N.—*a*, Red blood-corpuscles ; *b*, Bundles of fibrin fibres. See page 229.

PLATE X

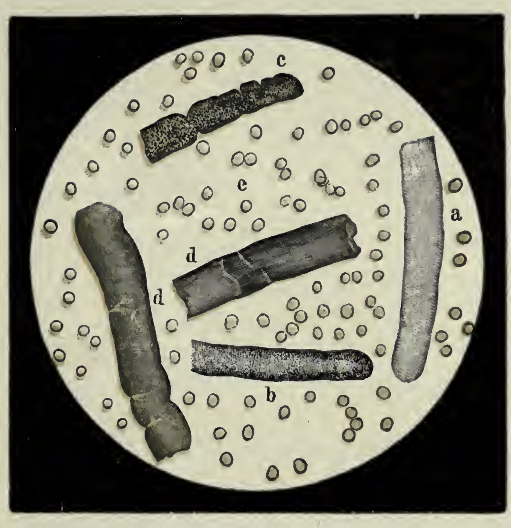

FIG. O.—*a*, Hyaline cast; *b*, Finely granular cast; *c*, Coarsely granular cast; *d*, Waxy cast; *e*, Red blood-corpuscles. See page 230.

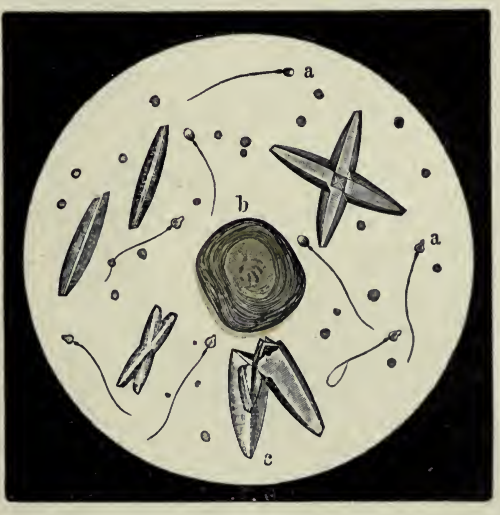

FIG. P.—*a*, Spermatozoa; *b*, Prostatic corpuscles (*Corpora amylacea*); *c*, Spermin crystals. See page 235.

PLATE XI

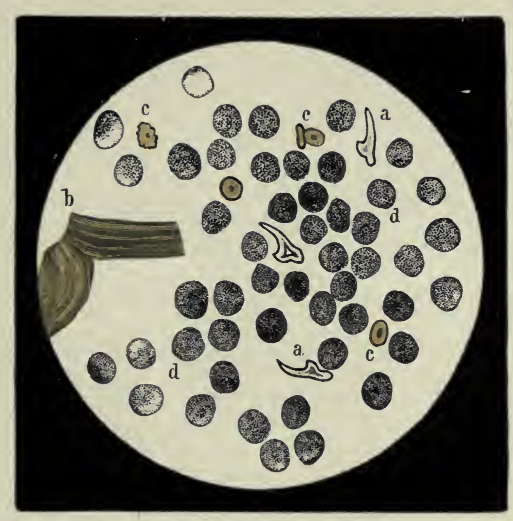

FIG. Q.—*a,* Echinococcus hooklets; *b,* Echinococcus membrane; *c,* Red blood-corpuscles; *d,* Leucocytes. See page 235.

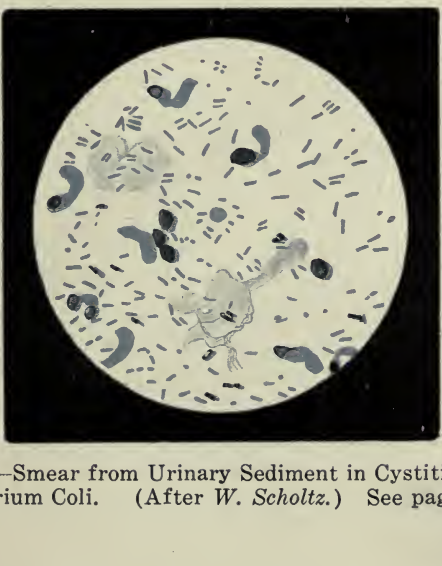

FIG. R.—Smear from Urinary Sediment in Cystitis due to Bacterium Coli. (After *W. Scholtz.*) See page 239.

PLATE XII

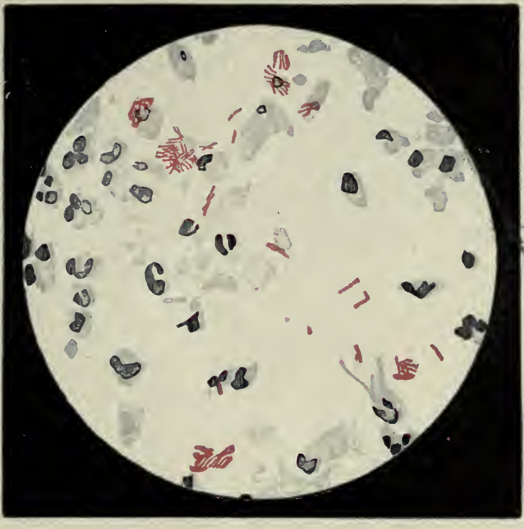

FIG. S.—Tubercle Bacilli in the Urine in Tuberculosis of the Bladder. (After *W. Scholtz.*) See page 240.

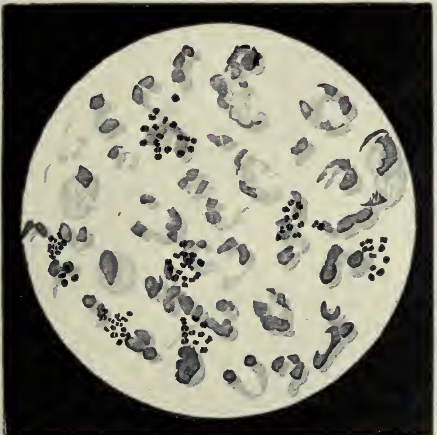

FIG. T.—Gonococci in Urethral Secretion. (After *W. Scholtz.*) See page 247.

PLATE XIII

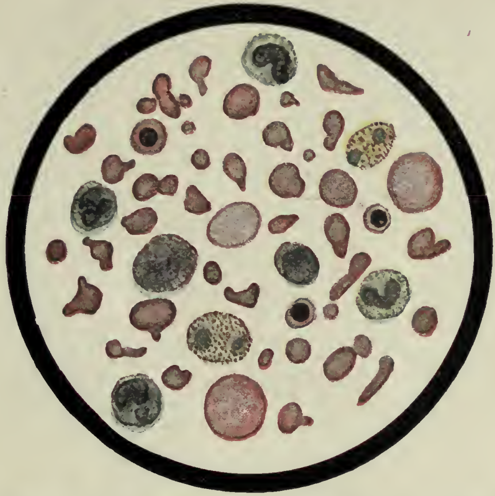

FIG. U.—Macrocytes, Microcytes, Poikilocytes, Erythroblasts, Normal Leucocytes. Triacid. (After *Grawitz.*) See page 262.

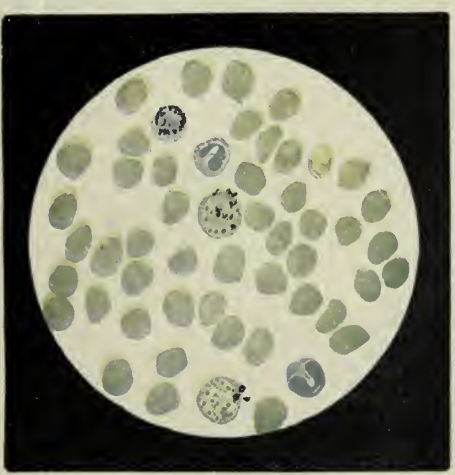

FIG. V.—Blood Smear made shortly before the Onset of a Tertian Chill. Parasite in the Process of Division. Stained according to *Manson.* (After *Kolle.*) See page 265.

PLATE XIV

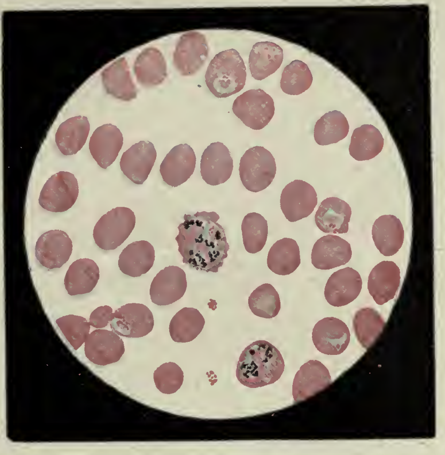

FIG. W.—Blood Smear taken during the Height of Tertian Fever. Large Rings and Full-grown Pigmented Parasites. Stained according to *Romanowski.* (After *Kolle.*) See page 265.

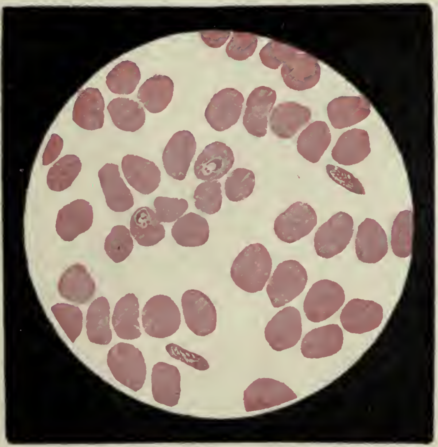

FIG. X.—Blood Smear taken from a Case of Chronic Tropical Malaria. Large Rings, Crescents. Stained according to *Romanowski.* (After *Kolle.*) See page 267.

PLATE XV

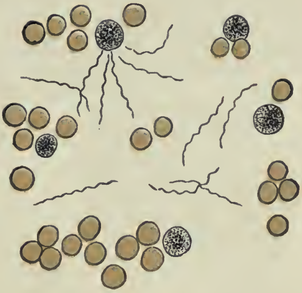

FIG. Y.—Spirilla of Relapsing Fever. (After *v. Jaksch.*)
See page 268.

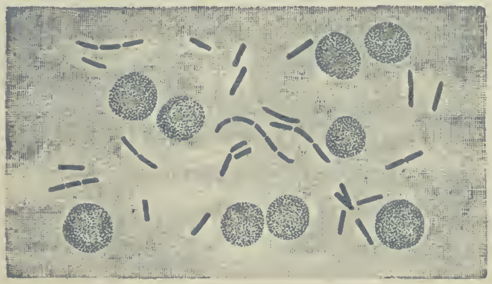

FIG. Z.—Anthrax Bacilli in Rabbit's Blood.
(After *v. Jaksch.*) See page 301.

PLATE XVI

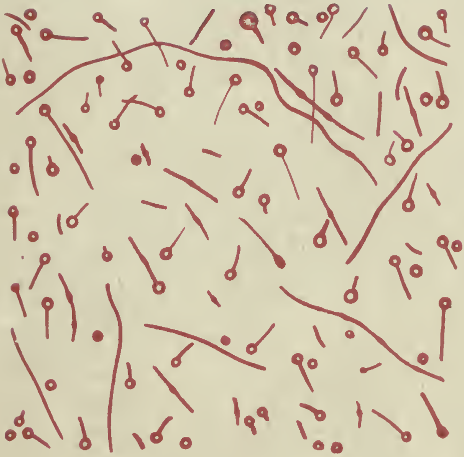

FIG. a.—Tetanus Bacilli (pure culture). (After *v. Jaksch.*)
See page 302.

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